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# Chilling sensitivity of *Arabidopsis thaliana* with genetically engineered membrane lipids

Claim 65
Glycerol-3-phosphate acyltransferase

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**Upon transfer of a genetically engineered *Escherichia coli* gene for glycerol-3-phosphate acyltransferase (*plsB*) to *Arabidopsis thaliana* (L.) Heynh., the gene is transcribed and translated into an enzymatically active polypeptide. This leads to an alteration in fatty acid composition of membrane lipids. From these alterations it is evident that the enzyme is located mainly inside the plastids. The amount of saturated fatty acids in plastidial membrane lipids increased. In particular, the fraction of high-temperature melting species of phosphatidylglycerol is elevated. These molecules are thought to play a crucial role in determining chilling sensitivity of plants. An increase in sensitivity could be observed in the transgenic plants during recultivation after chilling treatment. Implications for the hypothesis of phosphatidylglycerol-determined chilling sensitivity are discussed.**

*Key words:* acyltransferase/fatty acid composition/phosphatidylglycerol/stress/transgenic plant

## Introduction

Many plants of tropical and subtropical origin, when exposed to low non-freezing temperatures (0–15°C), develop symptoms indicative of injured performance that are collectively named chilling sensitivity. The critical temperatures may interfere in many functions with consequences for growth, yield, transport and storage in such plants (Long and Woodward, 1988). The phenomenon always includes impairment exceeding that resulting from the reduction of metabolic rates caused by decreasing temperatures. The metabolic interference at low temperatures may also limit growth or survival after subsequent return to higher temperatures. Maintenance of a fluid state of membrane lipid components is thought to be one of the prerequisites for unimpaired survival at lower temperature (Lyons, 1973). A lateral phase separation of non-fluid membrane components may be the primary defect caused by exposure to low temperature.

Chilling sensitivity in different plants may be mediated by different membrane systems depending on their content of specific, non-fluid lipid classes. In plastidic membranes phosphatidylglycerol (PG) has been identified as a critical component in this respect (Murata, 1983). The correlation between chilling sensitivity and a high content of non-fluid PG has been exemplified in many plants (Roughan, 1985; Bishop, 1986). The phase transition of this high-temperature melting fraction of phosphatidylglycerol (htm-PG) is the first event to occur as temperature drops which then initiates membrane dysfunction (Raison and Wright, 1983; Murata and Yamaya, 1984; Bishop and Kenrick, 1987). On the other hand, a low proportion of htm-PG is required but not sufficient for chilling tolerance, since additional factors are essential for membrane function at low temperature (Long and Woodward, 1988).

In chilling tolerant plants, all these requirements are met and, therefore, defined factors can be experimentally perturbed. In the experiments described here, we have increased the proportion of saturated fatty acids in PG in chloroplasts and thus increased its non-fluid proportion. This caused a chilling tolerant plant to become chilling sensitive with symptoms visible during recultivation of cold-treated plants under warm conditions. In parallel work published recently (Murata *et al.*, 1992) a similar strategy was followed and despite the use of different plant species and enzymes both approaches came to the same conclusions and, therefore, provide independent evidence for the involvment of PG in chilling sensitivity.

The biosynthesis of the non-fluid PG component in plastids (Mudd *et al.*, 1987) can be attributed to the properties of a single enzyme, acyl-ACP:*sn*-glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15). This acyltransferase initiates glycerolipid synthesis in plastids by catalysing the acylation of the C1 position of *sn*-glycerol-3-phosphate. In chilling tolerant plants, the acyltransferase selects only oleic acid (18:1) from the mixture of acyl-ACP containing mainly 18:1 and palmitic acid (16:0) (Frentzen *et al.*, 1983). In contrast, in chilling sensitive plants (Cronan and Roughan 1987; Frentzen *et al.*, 1987), the enzyme indiscriminately accepts all acyl groups of acyl-ACP. The product 1-acyl-glycerol-3-phosphate is subsequently esterified at C2 with 16:0 in both chilling tolerant and sensitive plants (Frentzen *et al.*, 1983). The resultant phosphatidic acid is converted to PG (Mudd *et al.*, 1987) in which the fatty acids reflect the selectivity of the first acyltransferase: chilling resistant plants produce mainly 18:1/16:0 PG, whereas chilling sensitive plants have in addition various proportions of 16:0/16:0 PG, depending on the selectivity of the particular acyltransferase. After assembly, C1-bound 18:1 is converted to linoleic (18:2) and further to linolenic acid (18:3). The C2-bound 16:0 is partially converted to 3-*trans*-hexadecenoic acid (*trans*-16:1), which in its physical properties is similar to a saturated fatty acid (Bishop and Kenrick, 1987; Murata and Yamaya, 1984; Raison and Wright, 1983). Therefore, it is the lack of selectivity of the first acyltransferase which results in the accumulation of high-temperature melting 16:0/16:0 and 16:0/*trans*-16:1 species of PG.

To increase the accumulation of htm-PG in a chilling tolerant plant, we transformed such plants in order to express

an acyltransferase that should increase the incorporation of 16:0 into the C1 position of glycerol-3-phosphate during lipid synthesis in chloroplasts. The acyltransferase from *Escherichia coli* encoded by the *plsB* gene (Lightner *et al.*, 1983) is well suited for this purpose, as it preferentially uses 16:0 for acylation (Rock *et al.*, 1981), and in fact, the transformation of a chilling tolerant plant with the *plsB* gene of *E.coli* resulted in increased chilling sensitivity.

## Results

### *Construction of a chimeric acyltransferase gene*

To achieve functional expression and correct subcellular targeting of the bacterial *plsB* gene we combined four different genetic elements. A strong plant promoter and the coding region of a signal sequence for import into plastids (transit peptide) were both isolated from rbcS-1, a strongly expressed gene of the small subunit of ribulose bisphosphate carboxylase (rubisco) of potato (Wolter *et al.*, 1988). This was fused to the coding region of the *plsB* gene from *E.coli* (Lightner *et al.*, 1983), and finally the polyadenylation signal from the octopine synthase gene was added (Koncz *et al.*, 1987). The fusion protein encoded by this chimeric construct starts with a transit peptide of 60 amino acids identical to that of rbcS-1 followed by the first three amino acids of the mature small subunit of rubisco. The following tryptophan codon specifies the fourth amino acid of the mature small subunit of rubisco as well as the fourth codon following the first of the two possible start codons of *plsB* (Lightner *et al.*, 1983). The *plsB* coding region continues for 803 codons.

### *Transformation of Arabidopsis*

The chimeric gene construct was cloned as a *Hin*dIII fragment into the unique *Hin*dIII site of pGSC1704 plasmid which was transferred to *Agrobacterium*. Cocultivation, selection and shoot induction of *Arabidopsis thaliana* were carried out as described earlier (Schmidt and Willmitzer, 1988). From two experiments six independent hygromycin resistant lines were regenerated. Plants of the fourth generation obtained by successive self-crossing were used for further analysis and in particular two lines, no.4 and no.6, were analysed in detail. Only plants from line no.4 exhibit a phenotype which differs significantly from wild type. These plants are tiny and prone to premature flowering. As a consequence no typical leaf rosette is formed and a multitude of shoots emerges from each plant. Plants of line no.6 are similar in habitus to wild type, but plants from both lines grow slowly and have a low yield of viable seeds.

### *Expression*

To demonstrate that the *plsB* sequence is actively transcribed total RNA was isolated from leaves of transgenic plants. After separation by gel electrophoresis and transfer to nylon filters the RNA was hybridized to a labelled probe of *plsB* antisense RNA (Figure 1). For both transgenic lines a signal of ~3 kb was observed, which is reproducibly more intensive in samples from line no.4. This size is in excellent agreement with the expected length for a transcript derived from the chimeric pHAMPL construct coding for 860 amino acids plus 5′ and 3′ non-translated regions.

To prove that this RNA is properly translated, proteins from crude membrane fractions were subjected to SDS–PAGE and transferred to nylon membranes. The *PlsB*

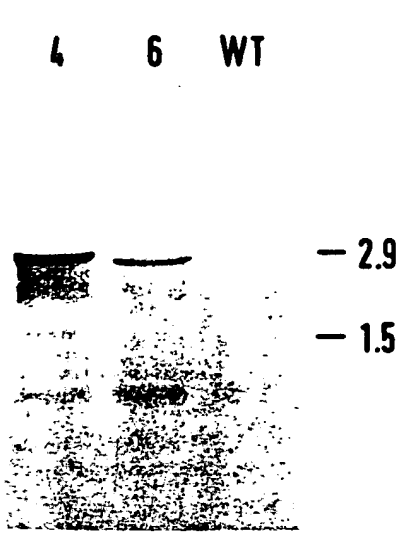


**Fig. 1.** Transcription of the bacterial acyltransferase gene in transgenic plants as analysed by Northern blotting. Identical quantities of total leaf RNA (15 μg) were subjected to electrophoresis, transferred to a nylon filter, and probed with a digoxigenin-labelled RNA complementary to a 1.2 kb fragment of the bacterial *plsB* gene. RNA from the transgenic lines no.4 and no.6 were loaded on to the left two lanes, RNA from wild type (WT) on to the right lane. The position and size of marker RNAs is indicated at the right.

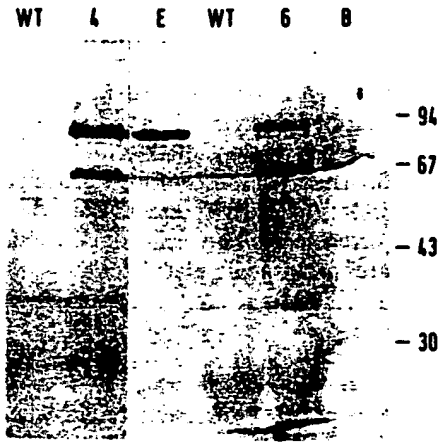


**Fig. 2.** Detection of the bacterial acyltransferase in transgenic plants by Western blotting. Identical quantities of membrane material corresponding to 20 μg chlorophyll were subjected to SDS–PAGE and probed with an antibody against the bacterial acyltransferase. Location and size of marker proteins are indicated at the right. WT, membranes from wild type plants; 4, membranes from transgenic line no.4; 6, membranes from transgenic line no.6; E, partially purified *E.coli* acyltransferase; B, sample buffer.

polypeptide was detected by anti-*PlsB* antibodies as shown in Figure 2. Lines no.4 and no.6 exhibit a positive reaction with the signal corresponding to a protein of ~90 kDa, which again agrees well with the expected molecular weight of the *plsB* protein. Weaker signals at lower molecular weight are probably due to degradation products.

Additionally, membrane fractions from wild type and transgenic plants were used for an enzymatic assay, and increased GPAT activity was observed in both transgenic lines. For line no.6 a 4-fold higher activity was measured as compared with control membranes, and in line no.4 the value was eight times higher. Therefore, we conclude that both transgenic lines transcribe the *plsB* gene and translate

**Table I.** Lipid composition of wild type (WT) and transgenic *Arabidopsis* plants (lines no.4 and no.6)

| Lipid component | WT | no.4 | no.6 |
|---|---|---|---|
| Monogalactosyl diacylglycerol (MGD) | 40 | 39 | 39 |
| Digalactosyl diacylglycerol (DGD) | 16 | 14 | 15 |
| Sulfoquinovosyl diacylglycerol (SQD) | 3 | 4 | 3 |
| Cerebroside | 2 | 3 | 2 |
| Steryl glucoside | 2 | 3 | 1 |
| Phosphatidylcholine (PC) | 16 | 15 | 16 |
| Phosphatidylethanolamine (PE) | 7 | 7 | 7 |
| Phosphatidylglycerol (PG) | 9 | 9 | 10 |
| Phosphatidylinositol | 4 | 5 | 5 |
| Phosphatidylserine | <1 | <1 | <1 |
| Phosphatidic acid | <1 | <1 | <1 |

Data are given as mole %.

**Table II.** Fatty acid composition of the main lipid classes for wild type (WT) and transgenic plants (lines no.4 and no.6)

| | | Fatty acid | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lipid | Plant | 16:0 | 16:1[a] | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 |
| PG | WT | 34 | 21 | 0 | 1 | 2 | 8 | 8 | 26 |
| | no.4 | 56 | 17 | 0 | 1 | 4 | 3 | 4 | 16 |
| | no.6 | 53 | 17 | 0 | 0 | 4 | 3 | 5 | 17 |
| MGD | WT | 2 | 1 | 2 | 31 | 0 | 1 | 2 | 62 |
| | no.4 | 6 | 1 | 2 | 24 | 0 | 1 | 2 | 65 |
| | no.6 | 4 | 0 | 2 | 30 | 0 | 1 | 2 | 61 |
| DGD | WT | 16 | 0 | 1 | 2 | 2 | 2 | 4 | 73 |
| | no.4 | 23 | 0 | 1 | 1 | 3 | 1 | 3 | 69 |
| | no.6 | 30 | 0 | 1 | 2 | 3 | 1 | 2 | 60 |
| SQD | WT | 40 | 0 | 0 | 1 | 3 | 3 | 8 | 45 |
| | no.4 | 54 | 0 | 0 | 1 | 4 | 2 | 6 | 34 |
| | no.6 | 44 | 0 | 1 | 0 | 3 | 2 | 7 | 45 |
| PC | WT | 22 | 1 | 0 | 1 | 2 | 6 | 29 | 39 |
| | no.4 | 30 | 0 | 0 | 0 | 4 | 4 | 23 | 40 |
| | no.6 | 29 | 0 | 0 | 1 | 3 | 4 | 23 | 41 |
| PE | WT | 31 | 0 | 0 | 0 | 3 | 4 | 33 | 30 |
| | no.4 | 35 | 0 | 0 | 0 | 2 | 3 | 26 | 33 |
| | no.6 | 35 | 0 | 0 | 0 | 2 | 2 | 26 | 34 |

[a]16:1 in PG is *trans*-16:1.
For abbreviations see Table I. Fatty acids are characterized by carbon and double bond numbers. Data are given in mole %.

the RNA into an enzymatically active protein of the expected size. The expressed protein as well as the enzymatic activity are associated with membranes. The expression is higher in line no.4 as judged from Northern blots as well as from measured enzymatic activity.

### *Lipid composition*

The expression of an additional enzyme of bacterial origin which contributes to the synthesis of lipids, does not alter the overall proportions of membrane lipids in leaves, and in particular, the proportion of PG in chloroplasts was not significantly changed (Table I). A detailed analysis of the fatty acid composition in the different lipid classes and of their positional distribution in the main plastidial lipids revealed characteristic differences between the transgenic lines and wild type plants (Tables II and III). The data for wild type plants regarding lipid proportions as well as profiles and positional distribution of fatty acids are in good agreement with previously obtained figures (Browse *et al.*, 1986b, 1989a; Norman and St John, 1986; Kunst *et al.*, 1988, 1989). In transgenic plants the proportion of 16:0 is increased in all lipid classes to various extents. The increase is limited to the *sn*-1 position which is controlled by GPAT, and correlates with the proportion of the particular lipid class synthesized via the prokaryotic pathway within the plastid (Browse *et al.*, 1986a). Accordingly, the individual lipids show different changes in fatty acid profiles which will be detailed in the following with emphasis on the three major chloroplast lipids monogalactosyl diacylglycerol (MGD), digalactosyl diacylglycerol (DGD) and PG.

For MGD of line no.6 only small differences from wild type plants are seen which agrees with the weaker expression of the bacterial acyltransferase in these plants. In line no.4 a larger 16:0 increase was observed at *sn*-1, whereas in *sn*-2 C18 fatty acids were increased at the expense of C16 acids. This indicates an increase in eukaryotic MGD and may represent a compensatory effect. The additional 16:0 in *sn*-1 of MGD is not desaturated despite the fact that plants have this capacity as shown for spinach (Roughan *et al.*, 1987; Heemskerk *et al.*, 1991). As the increase in 16:0 is not

**Table III.** Positional distribution of fatty acids in the main plastidial lipid classes from wild type (WT) and transgenic plants (lines no. 4 and no. 6)

| Lipid | | Plant | 16:0 | 16:1[a] | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 |
|---|---|---|---|---|---|---|---|---|---|---|
| PG | *sn-1* | WT | 25 | 0 | 0 | 0 | 5 | 16 | 15 | 38 |
| | | no.4 | 61 | 0 | 0 | 0 | 6 | 5 | 7 | 21 |
| | | no.6 | 54 | 0 | 0 | 0 | 7 | 6 | 8 | 25 |
| | *sn-2* | WT | 55 | 37 | 0 | 1 | 1 | 1 | 2 | 4 |
| | | no.4 | 55 | 32 | 0 | 1 | 2 | 1 | 3 | 6 |
| | | no.6 | 56 | 35 | 0 | 1 | 2 | 1 | 2 | 5 |
| MGD | *sn-1* | WT | 3 | 0 | 1 | 2 | 1 | 2 | 3 | 88 |
| | | no.4 | 11 | 1 | 2 | 3 | 1 | 1 | 2 | 79 |
| | | no.6 | 7 | 1 | 2 | 3 | 1 | 1 | 2 | 84 |
| | *sn-2* | WT | 2 | 3 | 2 | 58 | 0 | 1 | 2 | 32 |
| | | no.4 | 3 | 1 | 2 | 36 | 0 | 0 | 3 | 56 |
| | | no.6 | 2 | 1 | 2 | 53 | 0 | 0 | 1 | 40 |
| DGD | *sn-1* | WT | 15 | 0 | 2 | 1 | 4 | 3 | 5 | 70 |
| | | no.4 | 37 | 0 | 2 | 1 | 5 | 2 | 3 | 50 |
| | | no.6 | 43 | 0 | 2 | 1 | 5 | 2 | 3 | 44 |
| | *sn-2* | WT | 17 | 0 | 1 | 5 | 2 | 2 | 2 | 71 |
| | | no.4 | 19 | 0 | 2 | 5 | 2 | 1 | 2 | 69 |
| | | no.6 | 24 | 0 | 2 | 3 | 1 | 1 | 2 | 67 |

[a]16:1 in PG is *trans*-16:1.
For abbreviations see Table I. Data are given as mole %.

balanced by increased desaturation of other fatty acids in prokaryotic MGD, this more saturated component may be subjected to an increased turnover resulting in its replacement by highly unsaturated eukaryotic MGD. A similar switching to eukaryotic MGD has been observed in *Arabidopsis* mutants which could neither synthesize nor desaturate this prokaryotic chloroplast lipid (Browse *et al.*, 1986a,b, 1989a; Kunst *et al.*, 1988, 1989). But compared with DGD and PG (see below), this increase of 16:0 in MGD is low. This could indicate that the desaturation level of this lipid is kept within narrow limits, whereas in PG and DGD higher levels of 16:0 can be tolerated.

In DGD of both transgenic lines the proportion of 16:0 in *sn*-1 is strikingly increased and higher than the proportion of the prokaryotic species which are characterized by C16 fatty acids at C2. This can be explained by the fact that eukaryotic DGD species always contain significant proportions of 16:0 in *sn*-1, and nearly all 16:0 found in the *sn*-1 position of wild type DGD has to be attributed to eukaryotic species. By substracting this value from that found in transgenic DGD it appears that a high proportion of the prokaryotic DGD is esterified with 16:0 in *sn*-1 as a consequence of the action of the bacterial acyltransferase. Future studies have to show whether 16:0/16:0 species of DGD do in fact accumulate in the transgenic plants, which could also contribute to chilling sensitivity. From studies with spinach chloroplasts it can be inferred that this completely saturated species represents a major proportion of the prokaryotic DGD formed by isolated organelles (Heemskerk *et al.*, 1991). Similar considerations may apply to sulfoquinovosyl diacylglycerol (SQD) which also shows a rise in 16:0.

The most pronounced increase of 16:0 was found in PG which is of particular interest for several reasons. First, PG from chloroplasts is the only lipid class of this organelle which is exclusively synthesized *de novo* within plastids and accordingly has a purely prokaryotic diacylglycerol moiety. Therefore, its fatty acid composition will monitor changes of the biosynthetic activity within these organelles.

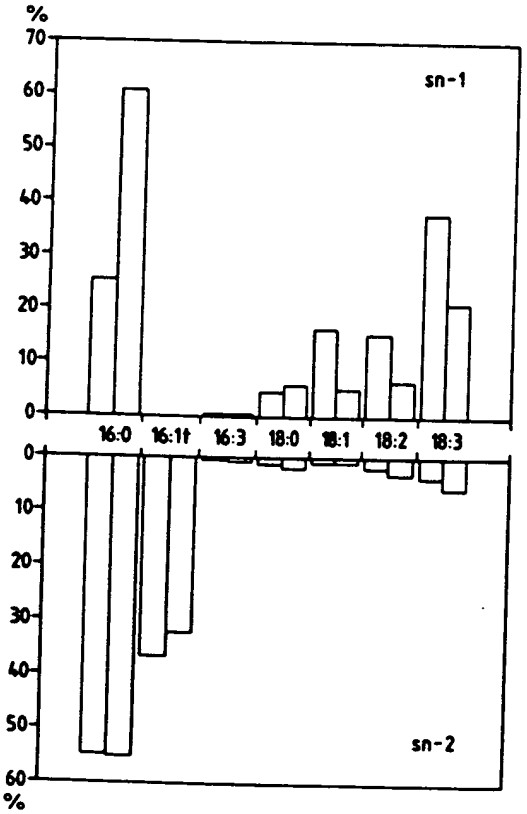


**Fig. 3.** Positional distribution of fatty acids in PG from wild type and transgenic *Arabidopsis thaliana* (line no.4) plants. Fatty acid profiles (mole %) are given for the *sn*-1 and *sn*-2 positions of the glycerol backbone. Fatty acids are characterized by carbon and double bond numbers. 16:1t stands for 3-*trans*-hexadecenoic acid which is confined to the C2 position of chloroplast PG. For each fatty acid the left bar represents the proportion from wild type and the right bar the proportion from transgenic plants.

Furthermore, in leaves most of the PG is localized in plastids as is evident from the low proportion of C18 fatty acids in the *sn*-2 position of leaf PG (Dorne and Heinz, 1989;

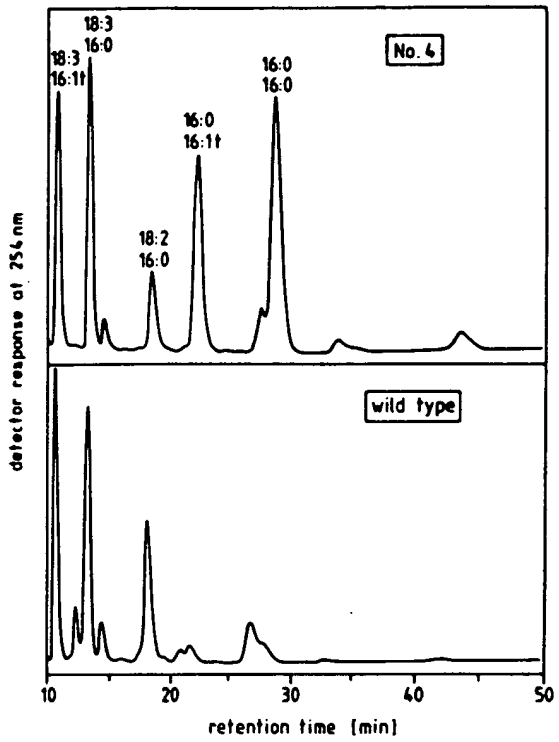


Fig. 4. HPLC chromatogram showing the composition of molecular species of PG from wild type and transgenic *Arabidopsis* plants (line no.4). Individual components are identified by their constituent fatty acids with the fatty acid most likely in the *sn*-1 position given above the *sn*-2-bound acyl group.

Table III). Finally, the fatty acid composition of PG and in particular the proportion of non-fluid components as outlined above have been proposed to be one of the factors limiting chilling tolerance.

In PG the 16:0 content was raised from ~35% in wild type to over 50% in the two transgenic lines (Table II). The positional analysis reveals that this increase is confined to the *sn*-1 position, where the proportion of 16:0 is more than doubled. Because of their importance for chilling sensitivity the data of the positional distribution of fatty acids in PG are separately depicted in Figure 3 which in addition show convincingly that the engineering is limited to the *sn*-1 position without any interference at C2.

The fact that the *sn*-2 position of PG is almost completely esterified by 16:0 and *trans*-16:1 (see Table III) in combination with the increase of 16:0 in *sn*-1 of PG in the transgenic lines suggest that a high proportion of hmt-PG with two rigid fatty acids such as 16:0, 18:0 or *trans*-16:1 combined in one lipid molecule should be present in these plants. In Figure 4 the resolution of the molecular species of PG from transgenic and wild type plants is shown. The two hmt species 16:0/*trans*-16:1 and 16:0/16:0 make up <5% of the PG species in the wild type plants, but are increased to >50% in the transformed line.

From the data of our lipid analysis we conclude that in the transgenic plants the correctly translated *PlsB* with its preference for 16:0 displays enzymatic activity leading to an alteration of lipid composition. The fact that the increase is almost limited to plastidial lipids is evidence that most of the enzymatically active *PlsB* is located within the plastids as a consequence of the leader sequence-facilitated import. The activity of *PlsB* redirects available fatty acids with regard to their positional location, and by this the total fatty acid composition is changed to some extent. By combining the data from Tables I and II the overall content of 16:0 can be estimated and an increase of ~50% from 14 mole % in the wild type to 22 mole % in the transgenic lines is calculated. Thus, the bacterial enzyme competes successfully not only with the native GPAT but also with the plastidial elongase and prevents part of the 16:0 from being converted to C18 fatty acids and from being desaturated at C2 of MGD.

### *Stress treatment*

The proportion of hmt-PG in the two transgenic lines exceeds that of many chilling sensitive plants. To test whether this alteration is sufficient to create chilling sensitivity, transgenic as well as wild type plants were incubated at 4°C in the dark. Even after prolonged exposure no significant difference between transgenic and wild type plants was visible. This changed drastically when plants kept for 7 days at 4°C were returned to 20°C. While wild type plants were apparently unaffected by this treatment, the transgenic plants showed wilting in their older leaves which after 2 days became brown and necrotic as shown in Figure 5. Recent data show that incubation of the transgenic plants at low temperature under illumination leads to even more severe symptoms, as plants then started wilting after 3 to 4 days and finally died (data not shown).

The observed increase in chilling sensitivity might be a non-specific decrease in stress tolerance due to a reduced fitness of the transgenic plants. To test this, plants were subjected to a heat stress experiment. Transgenic as well as wild type plants withstand a 2 h treatment at 37°C without visible damage, but neither were able to survive the treatment at 42°C, as described for wild type *Arabidopsis* plants (Binelli and Mascarenhas, 1990). Despite their limitation these results provide first evidence that the increased chilling sensitivity is not the consequence of a general and non-specific loss of stress tolerance, but may specifically be correlated with low temperature performance in dependence of PG fluidity.

## Discussion

We have isolated two independent transgenic *Arabidopsis* lines which express a modified bacterial gene in enzymatically active form. The newly introduced enzymatic activity changes the fatty acid composition of membrane lipids, and those components are most severely affected which are made inside the plastids. This implies that the new activity is targeted to the plastids and thus the N-terminal transit peptide of the rubisco small subunit gene is able to govern the import of this large *PlsB* protein consisting of more than 800 amino acids, which in addition is highly hydrophobic and in its native cell a membrane-bound component.

The transgenic lines show an increase of 16:0 in the *sn*-1 position of nearly all lipids, but this rise is most prominent in those lipids which are synthesized inside the plastid. Whether the slight increase in 16:0 observed in extraplastidic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is due to residual extraplastidial activity or has to be ascribed to secondary effects such as export from plastids (Browse *et al.*, 1989b) cannot be answered at present. Alternatively, those 16:0 molecules which are released from C1 of MGD

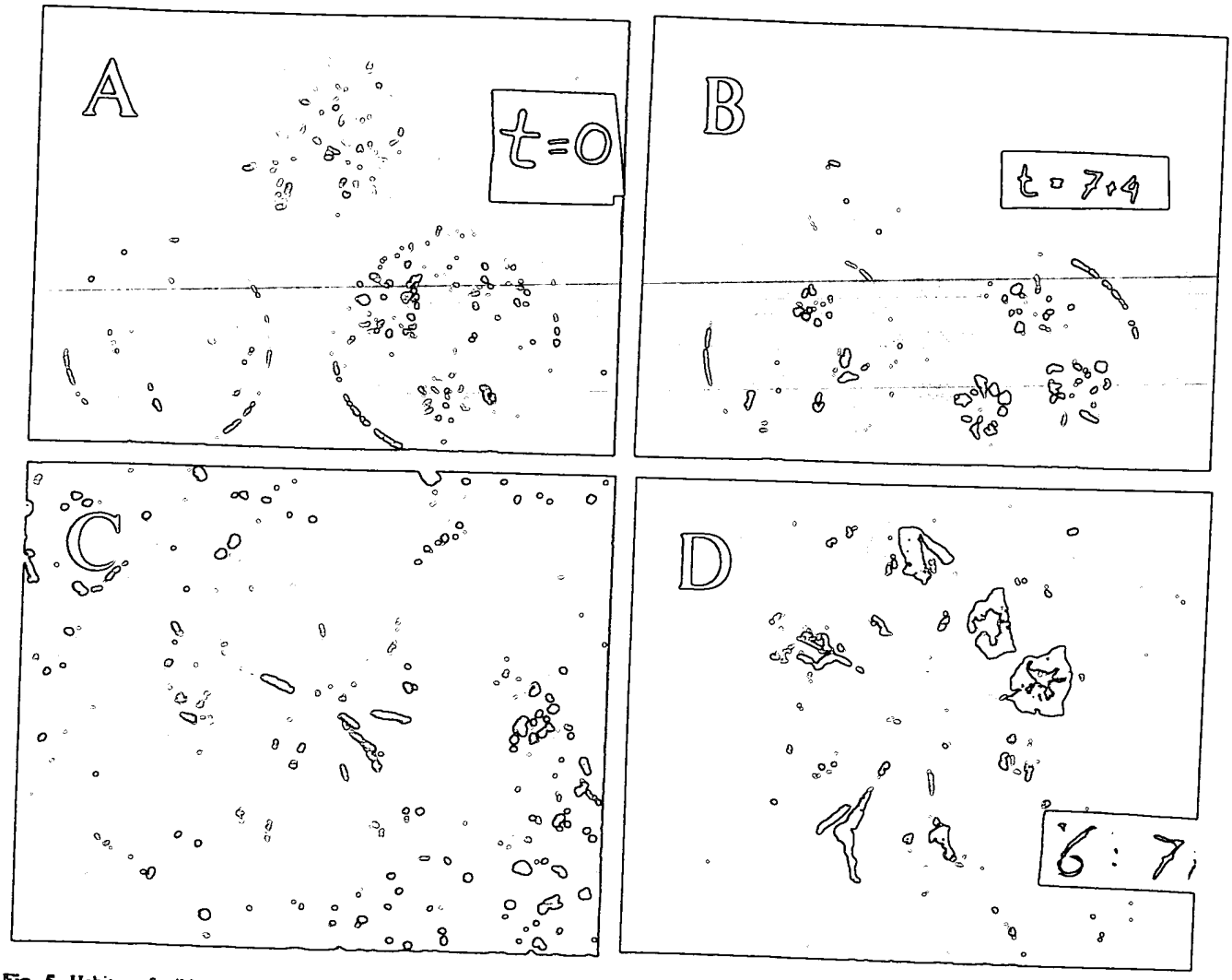


Fig. 5. Habitus of wild type and transgenic plants before and after chilling treatment and reculture. Plants are shown before (A and C) and after (B and D) they were kept for 7 days at 4°C and an additional 4 days at 20°C. Pots in (A) and (B): top, wild type; bottom left, line no.4; bottom right, line no.6. (C) and (D): the same plant of line no.6 before and after cold treatment in detail.

by enhanced turnover as discussed above may be exported from the plastids into the endoplasmic reticulum and thus lead to a higher incorporation into the *sn*-1 position of extraplastidic lipids.

The high incorporation of 16:0 into *sn*-1 of PG increased the hmt-PG species to such an extent that according to the hypothesis of PG-dependent chilling sensitivity these transgenic plants have to be classified as potentially chilling sensitive. Our *Arabidopsis* plants with the altered lipid composition respond to a cold treatment by developing symptoms of leaf necrosis during recultivation at normal temperature. A retardation in the development of symptoms is often observed in plants with natural chilling sensitivity (Salveit and Morris, 1990), and this delayed response was considered as arguing against the hypothesis of PG-dependent chilling sensitivity, since phase transitions are rapid processes and one would expect an immediate effect on the plant. But the observation of the same delay in damage in our transgenic plants supports the view of a causal correlation between hmt-PG and chilling sensitivity.

On the other hand, our transgenic plants have not only an altered lipid composition, but also a new foreign membrane protein in the plastids. One could argue that the mere presence of this protein might induce the chilling sensitivity and the altered phenotype of our plants. But the data from an independent experiment (Murata *et al.*, 1992) support the conclusion that it is not just the protein, but the consequence of its expressed activity which causes chilling sensitivity in the transgenic plants. In this study tobacco served as target plant and the acyltransferase from squash was used to increase 16:0 in PG. Its expression in transgenic plants led to similar changes in lipid compositions as observed in our studies and in particular, the symptoms of cold treatment became visible during subsequent recultivation under warm conditions and preferentially in older leaves. In contrast to the *E.coli* enzyme in our plants, the squash acyltransferase in tobacco is not membrane bound, but nevertheless the activities of both enzymes result in similar symptoms in two unrelated plant species. This strongly argues against a non-specific effect due to the presence of a foreign protein.

In both studies the increased incorporation of 16:0 is not limited to PG, and as no compensatory increase in desaturation is observed, the overall lipid saturation is increased as well. Therefore, it is not clear whether it is this reduced desaturation or the specifically increased proportion of hmt-PG which leads to chilling sensitivity. In this context recent experiments on the performance of desaturase mutants from *Arabidopsis* at chilling temperature are of particular

relevance (Hughly and Somerville, 1992). In these mutants the level of polyunsaturated fatty acids in plastidic lipids is strikingly reduced, whereas the saturated fatty acids in PG are not affected (Browse *et al.*, 1989a; Kunst *et al.*, 1989) and the proportion of hmt-PG is unchanged. When mutant plants of similar developmental state as used in our experiments were exposed to chilling temperatures, they did not show symptoms of chilling sensitivity. This argues in favour of a specific role of hmt-PG in initiating chilling sensitivity.

Apart from providing supporting evidence for the hypothesis of PG-mediated chilling sensitivity, our experiments have shown that it is possible to alter the composition of plant lipids by genetic engineering at specific points. This opens new access to physiological problems and has relevance for future activities in engineering other aspects of plant lipid metabolism.

## Materials and methods

### Materials

Plasmids: pCV701 (Koncz *et al.*, 1987), pH80 (8.0 kb *Hin*dIII fragment containing rbcS-1 cloned into pUC8) (Wolter *et al.*, 1988), pVL1 (Lightner *et al.*, 1983), pVV1300 (700 bp *Pst*I fragment containing *rbcS-c* in pUC8) (Wolter *et al.*, 1988), pGSC1704 (PGS, Ghent, Belgium).

Plant material: transgenic as well as control plants (*Arabidopsis thaliana*, ecotype Columbia-C24) were kept under permanent light at 20°C. All transgenic plants were germinated under selective conditions in order to ensure hygromycin resistance.

### Plasmid construction

For cloning and transformation of *E.coli*, standard techniques were used (Sambrook *et al.*, 1989). For constructing pHAMPL5 the promoter and coding region for the transit peptide from rbcS-1 were isolated as a 2.5 kilobase pair (kb) *Hin*dII–*Sph*I fragment. The coding region of *plsB* was isolated as a 2.5 kb *Bal*–*Stu*I fragment from pVL1 (Lightner *et al.*, 1983). These fragments were connected by insertion of a short *Sph*–*Bal*I fragment of 9 bp length isolated from rbcS-c, a rubisco cDNA clone (Wolter *et al.*, 1988). This short piece contained the processing site for the transit peptide. The *Stu*I site in pVL1 is located 87 nucleotides downstream from the stop codon of the *plsB* coding region and was fused to the 230 bp *Sal*–*Hin*dIII fragment from pCV701 (Koncz *et al.*, 1987) containing the polyadenylation site of the octopine synthase gene connected by the 15 bp *Sma*–*Sal*I linker from the pUC18 multiple cloning site.

### Raising antibodies against E.coli acyltransferase

To isolate an enriched *PlsB* protein fraction the *Eco*RI insert of pVL1 was transferred into pUC8. The increase in copy number leads to overexpression of the *plsB* gene (not shown). *PlsB* was partially purified following a published protocol (Green *et al.*, 1981). *Escherichia coli* cells harbouring the plasmid were grown overnight at 37°C under vigorous shaking in rich medium. All following steps were carried out at 0–4°C. After pelletting and washing in buffer A (50 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, pH 8.5) the cells were broken by ultrasonic treatment. Debris was removed by low speed centrifugation and the supernatant subjected to ultracentrifugation at 200 000 *g* for 60 min. The resulting pellet was resuspended in buffer B [25 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20% (w/v) glycerol, pH 8.5] and after adjusting Triton X-100 to 0.5% (w/v) stirred for ~30 min. After centrifugation at 200 000 *g* for 1 h the supernatant was collected and frozen at −70°C. Preparative SDS gel electrophoresis, immunization of rabbits and preparation of antiserum were carried out as described elsewhere (Weber *et al.*, 1991).

### RNA isolation and Northern blotting

Total RNA was prepared by a modification of the GTC procedure (Chirgwin *et al.*, 1979). For the detection of specific RNA molecules the DIG system (Boehringer, Mannheim) was used which needs labelled RNA as probes. For labelling, the 1.2 kb internal *Pvu*II fragment from *plsB* (bp 1429–2644 in pVL1) was cloned into the *Sma*I site of pBluescriptSK− (pATEC1200) and after linearization 1 μg DNA was used as template to transcribe a digoxigenin-labelled RNA using T7 polymerase.

About 15 μg of total RNA from transgenic and control plants were separated on a 2% agarose gel at 50 V for ~4.5 h. Electrophoresis was carried out according to standard conditions with minor modifications. The RNA was blotted on to a nylon membrane and fixed by UV treatment. Prehybridization, hybridization, washing and detection of the fluorescence signal followed manufacturer's instructions with some modifications (Düring, 1991).

### Isolation of membrane fractions and Western blotting

About 500 mg of fresh leaf material was frozen in liquid nitrogen and ground in a mortar to a fine powder. All following steps were carried out at 0–4°C. The powder was transferred to a small blender and after addition of 5 ml buffer A mixed in intervals for 12 s. After filtration through cheesecloth the membranes were pelleted for 10 min at 3000 *g*. The pellet was resuspended in 500 μl buffer B. Relative sample concentrations were based on chlorophyll content. Aliquots of resuspended membranes equivalent to 60 μg chlorophyll were used to measure enzymatic activity under standard conditions for 20 min at 24°C (Bertrams and Heinz, 1981). Proteins from membrane fractions were separated by standard SDS–PAGE (4.5% stacking gel, 10% separation gel: 30:0.8 acrylamide/bisacrylamide) for 1 h at 200 V. The proteins were transferred to a nylon membrane as described earlier (Weber *et al.*, 1991) with minor variations (1 $mA/cm^2$; transfer buffers contained 0.1% SDS to achieve mobilization of the large hydrophobic *PlsB* polypeptide). For detection of the *PlsB* protein the anti-*PlsB* antibody (dilution 1:500) was employed in the non-radioactive DIG system (Boehringer, Mannheim) with some modifications (Düring, 1991).

### Analysis of lipids and fatty acids

Before lipid extraction, the plant material was placed into boiling water for some minutes. Separation of lipid classes by thin layer chromatography (TLC), quantification of lipids by colorimetry and analysis of fatty acids as bromophenacyl esters have been described before (Haschke *et al.*, 1990). For a positional analysis, leaf lipids extracted from 2–3 g fresh weight were separated by TLC on two Kieselgel 60 plates in chloroform/methanol/acetic acid/water 91:30:4:4 (v/v). The individual compounds were recovered from the appropriate zone and subjected to enzymatic hydrolysis (*Rhizopus* lipase) followed by HPLC of fatty acids as *p*-bromophenacyl esters. Our analysis of wild type PG concurs with the previously determined pattern (Browse *et al.*, 1986a). For the analysis of molecular species from PG, diacylglycerol portions from PG (isolated from 2–3 g of fresh weight) were released enzymatically (phospholipase C) and converted to dinitrobenzoyl derivatives (Takamura *et al.*, 1986) for subsequent reverse-phase HPLC with isocratic elution (acetonitrile/isopropanol, 98:2, v/v, 3 μm RP18 ODS hypersil column, 0.46 × 12.5 cm). Identification was carried out by using reference species and by collecting individual peaks followed by analysis of constituent fatty acids.

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Claim 65
Superoxide dismutase

# Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase

(tobacco/photoinhibition/methyl viologen)

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**ABSTRACT** **Transgenic tobacco plants that express a chimeric gene that encodes chloroplast-localized Cu/Zn superoxide dismutase (SOD) from pea have been developed. To investigate whether increased expression of chloroplast-targeted SOD could alter the resistance of photosynthesis to environmental stress, these plants were subjected to chilling temperatures and moderate (500 μmol of quanta per $m^2$ per s) or high (1500 μmol of quanta per $m^2$ per s) light intensity. During exposure to moderate stress, transgenic SOD plants retained rates of photosynthesis ≈20% higher than untransformed tobacco plants, implicating active oxygen species in the reduction of photosynthesis during chilling. Unlike untransformed plants, transgenic SOD plants were capable of maintaining nearly 90% of their photosynthetic capacity (determined by their photosynthetic rates at 25°C) following exposure to chilling at high light intensity for 4 hr. These plants also showed reduced levels of light-mediated cellular damage from the superoxide-generating herbicide methyl viologen. These results demonstrate that SOD is a critical component of the active-oxygen-scavenging system of plant chloroplasts and indicate that modification of SOD expression in transgenic plants can improve plant stress tolerance.**

Oxygen is essential for the existence of aerobic life, but toxic oxygen species, which include the oxygen-centered superoxide ($O_2^-$), and hydroxyl (·OH), free radicals, as well as hydrogen peroxide ($H_2O_2$), are generated in all aerobic cells. Injury caused by these oxygen derivatives is known as oxidative stress (see refs. 1–3 for reviews). Superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) constitutes the first line of cellular defense against oxidative stress.

Oxidative stress is a major damaging factor in plants exposed to environmental stress, and there is strong evidence for the protective role of SOD in plants (see refs. 4 and 5 for reviews). Most plants contain a number of SOD isozymes that are located in various cellular compartments. In pea, Cu/Zn-containing SOD isoforms are found in chloroplasts and in the cytosol, whereas a Mn-containing enzyme is located in mitochondria (6). Tobacco has a more complex complement of SODs that includes at least five distinct isozymes (7, 8). Most notably, tobacco includes a distinct Fe-containing SOD in chloroplasts, as well as chloroplastic and cytosolic Cu/Zn SODs and mitochondrial Mn SOD.

Superoxide radicals are produced continuously in plant chloroplasts as $O_2$ is reduced to $O_2^-$ by electrons from the photosystems (the Mehler reaction) (4, 5). SOD converts $O_2^-$ to $H_2O_2$ that is then scavenged in chloroplasts by a series of oxidation/reduction reactions, known as the Halliwell–Asada pathway, that use ascorbate, reduced glutathione, and NADPH as electron donors (4).

The inhibition of photosynthesis that can occur when excess excitation energy reaches the reaction center is commonly referred to as photoinhibition. High light intensity, especially at extreme temperatures or water deficit, can cause increased electron flow to $O_2$, resulting in greater production of $O_2^-$ and $H_2O_2$. Although oxygen radicals appear to be involved in photoinhibition (9–11), the role of SOD in limiting the oxidative damage associated with photoinhibition has not been directly demonstrated (12, 13).

To investigate the possible protective functions of SOD in plant chloroplasts, we have developed transgenic tobacco plants that overexpress chloroplast-localized Cu/Zn SOD. These plants were analyzed for photosynthetic rate when exposed to light and temperature conditions that inhibit photosynthesis and for their ability to recover photosynthetic capacity after stress. Our results indicate that these transgenic plants have improved photosynthetic function at chilling temperatures and moderate light intensity, and they recover more effectively from severe stress than control plants. These changes correlate with increased resistance to oxidative damage caused by the herbicide methyl viologen (MV).

## MATERIALS AND METHODS

**Plant Transformation.** Chimeric gene constructs were developed to overexpress chloroplastic SOD subunit in plant cells (Fig. 1). Chloroplastic Cu/Zn SOD cDNA from pea (14, 15) was amplified by polymerase chain reaction using mutagenic primers that introduced an *Nco* I site at the translation start codon (ACATGG to CCATGG) and an *Xba* I site within the 3′ untranslated sequence. After digestion with *Nco* I and *Xba* I, this fragment was ligated into the *Nco* I and *Xba* I sites of the expression vector pRTL2 (a gift from J. R. Carrington, Department of Biology, Texas A&M University). This vector includes a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer and a CaMV 35S terminator sequence. The cDNA insert was fused at the translation initiation codon within the 5′ untranslated sequence of the tobacco etch virus (TEV) that provides highly efficient translational initiation. The completed chimeric gene cassette was excised with *Hin*dIII and ligated into the *Hin*dIII site of the binary shuttle vector pBIN 19 (16) and mobilized to *Agrobacterium tumefaciens* strain LBA 4404 by triparental mating. Transformation of tobacco leaf disks was performed according to Horsch *et al.* (17), and >20 putative transgenic plants were regenerated.

**SOD Isozyme Analyses.** Transgenic plants that expressed pea chloroplastic Cu/Zn SOD were identified by analysis of SOD isozymes in leaves, using a method described by Beauchamp and Fridovich (18) as modified by Bowler *et al.* (8). Leaf extracts from transgenic tobacco plants, untrans-

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Abbreviations: SOD, superoxide dismutase; MV, methyl viologen.
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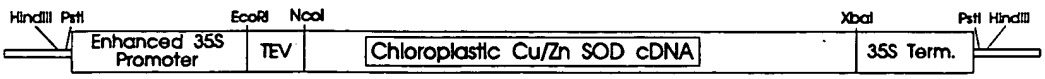


FIG. 1. Chimeric gene construct developed to overexpress pea chloroplastic SOD subunit in plant cells. This gene cassette was inserted into the binary plant transformation vector pBIN 19 and introduced into plant cells by *Agrobacterium*-based transformation. See text for further description.

formed tobacco plants (Xanthi), and pea plants were electrophoresed in nondenaturing polyacrylamide gels and negatively stained for SOD activity. Intensities of pea Cu/Zn SOD bands and native tobacco SOD bands on activity gels were estimated with a Molecular Dynamics scanning laser densitometer system (provided by the Texas Tech University Biotechnology Institute). Analysis of SOD isozymes extracted from crude chloroplast preparations of leaves of transgenic tobacco plants was also performed to determine whether the transgene product was correctly targeted to tobacco chloroplasts. Briefly, 1 g of leaf tissue was ground gently in 10-ml aliquots of 20 mM Hepes, pH 7/0.4 M sorbitol/2 mM $MgCl_2$ and the slurry was filtered through a 20-$\mu$m mesh to remove unbroken cells. Chloroplasts were pelleted by centrifugation at 200 × *g* and washed in the same buffer. Extracts were analyzed by SOD activity gel as described above. Two independently transformed plants that expressed the introduced gene at highest levels were self-pollinated, and the offspring of these plants (hereafter referred to as transgenic SOD plants) were grown in a greenhouse and used for our analyses.

**Analysis of Photoinhibition.** Net photosynthetic rates were measured for transgenic SOD plants and untransformed plants (Xanthi) by measuring $O_2$ evolution from 10-$cm^2$ leaf disks with a gas-phase, $O_2$ electrode system (Hansatech Instruments, Pentney, King's Lynn, U.K.) under saturating $CO_2$. Leaf disks were subjected independently to either moderate or severe stress regimes. Control leaf disks of each genotype were maintained at 25°C and the light intensity of the stress treatments for the duration of the experiments. To ensure that measurement of $O_2$ evolution accurately represented photosynthesis, rates of $^{14}CO_2$ fixation were also determined. Since both measurements correlated closely in all cases, only the $O_2$ evolution data are presented.

*Moderate stress treatment.* Leaf disks from transgenic SOD plants and Xanthi plants were equilibrated at 500 $\mu$mol of quanta per $m^2$ per s and 25°C for 1 hr, after which photosynthetic rates were determined at 470 $\mu$mol of quanta per $m^2$ per s and 25°C. Disks were then chilled to 10°C at 500 $\mu$mol of quanta per $m^2$ per s on a cooling block. Leaf disks were removed at designated intervals and photosynthetic rates were measured at 470 $\mu$mol of quanta per $m^2$ per s and 10°C.

*Severe stress treatment.* Leaf disks were equilibrated at 1500 $\mu$mol of quanta per $m^2$ per s and 25°C for 1 hr. Photosynthetic rates were determined at 975 $\mu$mol of quanta per $m^2$ per s and 25°C. The disks were then rapidly cooled to 3°C at 1500 $\mu$mol of quanta per $m^2$ per s and kept under these conditions for 4 hr. After this treatment, photosynthetic rates measured at 975 $\mu$mol of quanta per $m^2$ per s and 3°C were $<1$ $\mu$mol of $O_2$ per $m^2$ per s for all leaf disks. Leaf disks were then quickly warmed to 25°C, and $O_2$ evolution was monitored at 975 $\mu$mol of quanta per $m^2$ per s continuously until steady-state photosynthetic rates were reached (within 30 min for both transgenic SOD and Xanthi leaf disks).

**MV Treatment.** MV damage was analyzed as described by Bowler *et al.* (8) with modifications. Leaf disks (1.5 $cm^2$) collected from transgenic SOD plants and untransformed Xanthi plants were transferred to 3.5-cm Petri dishes containing 3 ml of MV solutions at various concentrations. Samples were vacuum infiltrated for 5 min and incubated at 21°C for 16 hr in darkness. Leaf disks were then illuminated (500 $\mu$mol of quanta per $m^2$ per s) for 2 hr, then incubated in darkness at 30°C for an additional 16 hr.

*Cell leakage analysis.* The conductivity of the decanted MV solution was measured with an Orion model 120 conductivity meter. The MV solutions were recovered from the conductivity meter cell and autoclaved, with the damaged leaf disks, for 15 min to release all solutes. The conductivity of the MV solution was again determined and the percentage of electrolyte leakage attributable to MV treatment was determined by dividing the conductivity value of the test sample by the conductivity of the sample after autoclaving (100% electrolyte leakage).

*Pheophytin measurements.* Pheophytin is a breakdown product of chlorophyll that results from the loss of the Mg moiety. After MV treatment, pigments were extracted from leaf disks in 80% acetone and the percentage of chlorophyll converted to pheophytin was determined by an increase in absorbance at 553 nm relative to absorbance at 665 nm (8, 19).

## RESULTS

Analysis of SOD isoforms from leaves of two transgenic SOD plants that accumulated significant amounts of pea chloroplastic Cu/Zn SOD is shown in Fig. 2. Densitometric analysis of SOD activity gels indicated that transgenic SOD plants accumulated the pea SOD isoform to levels ≈2-fold higher than that of endogenous chloroplastic Fe SOD or Cu/Zn SODs in untransformed tobacco plants. Notably, native Cu/Zn SOD isoforms were not detectable in any transgenic SOD plants that expressed detectable amounts of pea chloroplastic Cu/Zn SOD, indicating that expression of the introduced Cu/Zn SOD gene somehow interfered with the activity of native tobacco Cu/Zn SOD isoforms. Pea Cu/Zn SOD was detected in chloroplasts isolated from transgenic plants (Fig. 2), indicating that this enzyme was incorporated into tobacco chloroplasts.

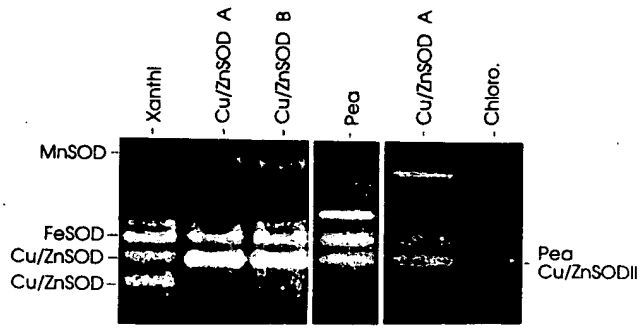


FIG. 2. Samples (25 $\mu$g) of total protein from leaf extracts from Cu/Zn SOD transgenic tobacco plants, untransformed tobacco plants (Xanthi), and pea plants were electrophoresed in nondenaturing polyacrylamide SOD activity gels. Native tobacco isoforms are indicated at left. Mn SOD is located in mitochondria. Fe SOD is found in chloroplasts, as is the high-mobility Cu/Zn SOD isoform (bottom band). The other (slower) Cu/Zn SOD isoform is found in the cytosol. Pea isoforms include (from top to bottom) mitochondrial Mn SOD, cytosolic Cu/Zn SOD I, and chloroplastic Cu/Zn SOD II. Note that the native tobacco chloroplastic Cu/Zn SOD isoform was not detectable in the extracts of transgenic leaves. Chloroplasts isolated from transgenic SOD plants (Chloro.) contain bands that correlate with native Fe SOD and pea Cu/Zn SOD II.

Photosynthetic rates determined by $O_2$ evolution from leaf disks of Xanthi plants and transgenic SOD plants before photoinhibitory treatments were virtually identical, averaging 35 $\mu$mol of $O_2$ per $m^2$ per s (Fig. 3). When leaf disks of Xanthi plants were exposed to moderate stress (10°C, 500 $\mu$mol of quanta per $m^2$ per s), their photosynthetic rates decreased by ≈95% to a mean of 1.75 $\mu$mol of $O_2$ per $m^2$ per s. However, under identical conditions, photosynthetic rates of transgenic SOD leaf disks were reduced by only 75%, to a mean of 8.75 $\mu$mol of $O_2$ per $m^2$ per s. Although reduced photosynthesis was observed in leaf disks of both genotypes during moderate stress conditions, leaf disks of transgenic SOD plants exhibited higher photosynthetic capacity than Xanthi leaf disks. It should be noted that net photosynthetic rates of both Xanthi and transgenic SOD leaf disks rapidly recovered after moderate stress exposure when they were warmed to 25°C and maintained at 50 $\mu$mol of quanta per $m^2$ per s for 30 min (Fig. 3). This indicates that, under these moderate stress conditions, little if any long-term oxidative damage had occurred in leaf disks of either genotype.

Since complete recovery of photosynthesis was observed in leaf disks of both Xanthi and transgenic SOD plants after moderate stress, increased stress levels were necessary to determine whether differences existed in their capacity to cope with and to recover from more severe stress. After exposure to 1500 $\mu$mol of quanta per $m^2$ per s and 3°C for 4 hr, photosynthetic rates for both Xanthi and transgenic SOD leaf disks were <1 $\mu$mol of $O_2$ per $m^2$ per s. When warmed to 25°C and measured at 975 $\mu$mol of quanta per $m^2$ per s, photosynthesis in leaf disks from Xanthi plants recovered, within 30 min, to a mean steady-state rate of 12 $\mu$mol of $O_2$ per $m^2$ per s (Fig. 4). Thus, after severe stress, the photosynthetic capacity of Xanthi leaf disks was only 36% of that measured before stress treatment, indicating that substantial oxidative damage that could not be rapidly reversed had occurred. Under the same conditions, photosynthetic rates of leaf disks from transgenic SOD plants recovered to a mean steady-state rate of 31 $\mu$mol of $O_2$ per $m^2$ per s. Hence, transgenic SOD leaf disks retained photosynthetic capacity after severe stress that was nearly 90% of that before stress exposure, indicating that little oxidative damage had occurred. Although leaf disks of both Xanthi and transgenic SOD plants exhibited complete inhibition of photosynthesis during exposure to severe stress conditions, most of this inhibition was rapidly reversed in transgenic SOD leaf disks,

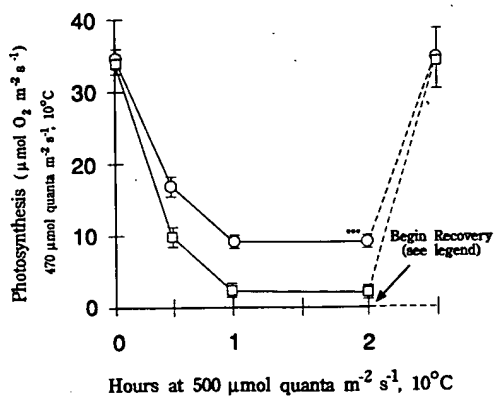


FIG. 3. Net photosynthetic rates were measured for transgenic SOD plants (○) and untransformed Xanthi plants (□) during moderate photoinhibitory stress (500 $\mu$mol of quanta per $m^2$ per s and 10°C). After stress treatment, leaf disks were allowed to recover for 30 min at 25°C and 50 $\mu$mol of quanta per $m^2$ per s. Photosynthetic rates for control leaf disks of each genotype maintained at 25°C and 500 $\mu$mol of quanta per $m^2$ per s did not change for the duration of the experiment. Values are means ± SD ($n$ = 10–12 plants of each genotype). ***, $P$ = 0.001.

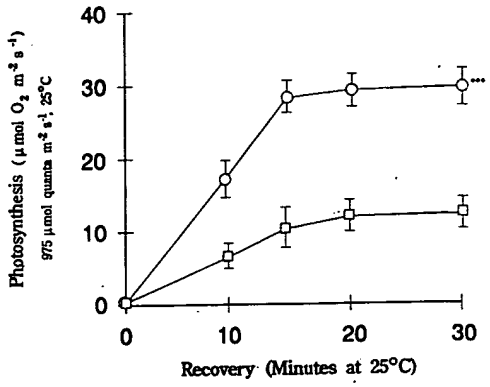


FIG. 4. Net photosynthetic rates were measured for transgenic SOD plants (○) and untransformed Xanthi plants (□) at 25°C after exposure to severe photoinhibitory stress (1500 $\mu$mol of quanta per $m^2$ per s, 3°C for 4 hr). Photosynthetic rates for control leaf disks of each genotype maintained at 25°C and 1500 $\mu$mol of quanta per $m^2$ per s did not change for the duration of the experiment. Values are means ± SD ($n$ = 10–12 plants of each genotype). ***, $P$ = 0.001.

indicating that they had suffered substantially less damage than Xanthi leaf disks. These results demonstrate that significant reduction in the levels of photoinhibitory stress can be achieved in leaves of transgenic tobacco plants that overexpress pea chloroplast Cu/Zn SOD.

To directly correlate the enhanced photosynthetic performance of transgenic SOD leaves under chilling and high light intensity with increased resistance to oxidative stress, leaf disks from transgenic SOD and Xanthi plants were treated with MV, a contact herbicide that causes massive, light-mediated accumulation of $O_2^-$ in photosynthetic tissues (20) (Fig. 5). The extent of cellular damage was quantified by solute leakage, which is a measure of membrane disruption (8). Leaf disks of Xanthi plants showed a dose-dependent increase in membrane damage, reaching nearly complete disruption (≈90% of maximum solute leakage) at 2.4 $\mu$M MV. Tissues of transgenic SOD plants showed significantly less damage at 0.6 and 1.2 $\mu$M MV than Xanthi tissues. However, at 2.4 $\mu$M MV the extent of damage in transgenic leaf disks was not significantly different from that of control samples. These observations were extended by analysis of pheophytin production in MV-treated leaf disks as a measure of chlorophyll damage (Fig. 6). Chlorophyll damage in transgenic SOD leaf disks at 1.2 $\mu$M MV was reduced by an average of 40% compared with Xanthi leaf disks, but at 2.4 $\mu$M MV, much less difference was seen. These results confirm that transgenic SOD plants exhibit increased resistance to MV-

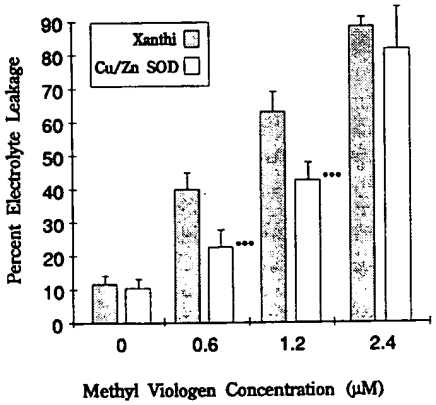


FIG. 5. Analysis of cellular damage in MV-treated leaf disks of transgenic SOD and untransformed Xanthi plants by measurement of percent solute leakage in treated tissues compared with autoclaved tissues. Values are means ± SD ($n$ = 9 plants of each genotype). ***, $P$ = 0.001.

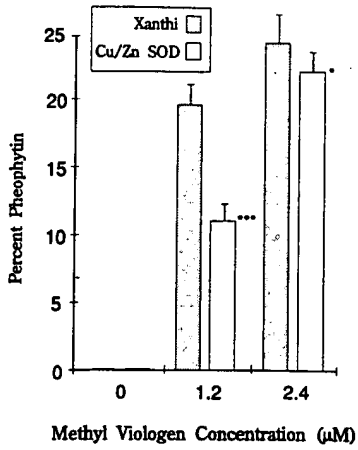


FIG. 6. Analysis of oxidative damage in MV-treated leaf disks from transgenic SOD plants and Xanthi plants by measurement of the conversion of chlorophyll to pheophytin. Values are means ± SD ($n$ = 5 plants of each genotype). ***, $P = 0.001$; *, $P = 0.05$.

mediated oxidative damage, although significant differences in MV tolerance between transgenic SOD plants and Xanthi plants could be detected only over a relatively narrow range of MV concentrations.

## DISCUSSION

The results presented clearly indicate that overexpression of pea chloroplast Cu/Zn SOD in tobacco leaves can improve their photosynthetic performance under moderate stress and maintain photosynthetic capacity after severe oxidative stress. We interpret these results to indicate that, under stressful conditions, transgenic SOD tissues have lower levels of $O_2^-$ than control tissues, leading to higher net rates of photosynthesis and reduced oxidative damage. Since it is unlikely that pea Cu/Zn SOD is inherently superior to tobacco chloroplast SOD isoforms, we believe that the difference in stress tolerance in transgenic SOD plants is directly related to increased levels of active SOD in their chloroplasts. These results do not necessarily indicate that $O_2^-$ causes chloroplast damage directly. Rather, rapid dismutation of $O_2^-$ in transgenic SOD plants could prevent its reaction with $H_2O_2$ to form highly reactive ·OH (8, 21). Increased levels of SOD and other oxidative stress response enzymes have been correlated with reduced photoinhibition in MV-resistant varieties of *Conyza bonariensis* (9) and the addition of exogenous SOD or catalase to thylakoids has also been shown to reduce photoinhibition (22). Our results show that direct manipulation of SOD gene expression alone can effect tolerance to photoinhibitory stress.

Since $O_2^-$ can dismutate without catalysis at a relatively high rate, one might question the need for increased SOD in plants. Spontaneous dismutation is highly pH-dependent, since it depends on protonation of $O_2^-$ to $HO_2$· with a $pK_a$ of 4.8 (23). Thus, under the alkaline conditions of the stroma of illuminated chloroplasts, it is likely that the rate of uncatalyzed dismutation of $O_2^-$ is at least 4 orders of magnitude lower than the $V_{max}$ of Cu/Zn SOD ($\approx 2 \times 10^9$ $M^{-1}s^{-1}$) (24) and is of little biological significance.

The higher photosynthetic rates for leaf disks from transgenic SOD plants compared with those for Xanthi plants under moderate low temperature stress (Fig. 3) are interesting, since both genotypes should differ only in their capacity to scavenge toxic oxygen species. Other genetically controlled factors that could cause differences in photosynthetic performance at low temperature are presumably the same in both genotypes. Therefore, the results presented here implicate the direct involvement of oxidative stress in the rapidly reversible reduction of photosynthesis at low temperature in tobacco.

Transgenic SOD plants also exhibited increased resistance to oxidative damage caused by exposure to low concentrations of MV, but at higher concentrations, this protective effect disappeared. Tepperman and Dunsmuir (25) were unable to detect any significant differences in resistance to MV between tobacco plants that expressed high levels (30- to 50-fold above normal SOD levels) of petunia chloroplast Cu/Zn SOD and control plants. Although the discrepancies between our results and those of this previous study can be largely explained by differences in assay methods, it is also possible that moderate increases in Cu/Zn SOD activity (as in our plants) can provide more effective protection from MV damage than very high SOD levels. In fact, Elroy-Stein *et al.* (26) have reported that moderate increases of Cu/Zn SOD provide MV resistance in human and mouse cells but large increases do not. Since Cu/Zn SODs are sensitive to end-product ($H_2O_2$) inhibition, it is possible that, when cells that express high levels of Cu/Zn SOD are treated with MV, they produce a burst of $H_2O_2$ that simply deactivates the enzyme.

The breakdown in stress resistance seen in our transgenic plants at 2.4 μM MV could indicate that the introduced Cu/Zn SOD is deactivated under these conditions. Bowler *et al.* (8) have reported significant protection from MV-induced oxidative damage in transgenic tobacco plants that overexpressed $H_2O_2$-insensitive, chloroplast-localized Mn SOD. Preliminary analysis in our laboratory indicates that tobacco plants that contain an analogous Mn SOD construct are substantially more resistant to high concentrations of MV than the transgenic Cu/Zn SOD plants described here (A.S.G., A.S.H., and R.D.A., unpublished results).

Further analyses of transgenic plants that overexpress chloroplast SODs will undoubtedly yield fundamental information about the effects of oxidative stress on chloroplasts, cells, and whole plants. Additional modifications of other components of the active-oxygen-scavenging system of plants will help to elucidate the interactions between these protective mechanisms. We remain hopeful that investigations of this type will eventually provide significant improvements in the tolerance of cultivated plants to environmental stress.

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Claim 65
Glutathione reductase

PHYSIOLOGIA PLANTARUM 77: 449–456. Copenhagen 1989

# Properties and functions of glutathione reductase in plants

Ivan K. Smith, Thomas L. Vierheller and Carol A. Thorne


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The assay and in vitro characterization of glutathione reductase (EC 1.6.4.2) is discussed. In vivo the $H_2O_2$-scavenging system in chloroplasts is the best documented role of reduced glutathione and glutathione reductase in plants. Similarly, reduction of $H_2O_2$, outside of the chloroplasts, requires glutathione and glutathione reductase; but the pathway, in terms of intermediates, is controversial. The notion that biological stress frequently causes cellular oxidation has lead to the suggestion that glutathione and glutathione reductase may play a role in stress resistance or tolerance mechanisms. The changes in glutathione reductase levels in response to low temperature, oxidative stress and drought are discussed.

*Key words* – $H_2O_2$ scavenging, plant stress, glutathione, glutathione reductase.

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## Introduction

Kosower and Kosower (1978) defined the glutathione status of cells as the total cellular concentration of glutathione and the relative distribution between its major forms: GSH, GSSG, and mixed disulfides such as GSS-protein. The idea that in most organisms GSH plays an essential metabolic role as a reductant is suggested by the relatively high concentration of glutathione (0.5 to 10 m*M*) in cells, the high ratio of GSH to GSSG (between 30 and 100 in animals), and the presence of GR (Rennenberg 1982, Ziegler 1985). The best example of a role for GSH and GR, under normal conditions, is their involvement in the $H_2O_2$-scavenging pathway in chloroplasts (Foyer and Halliwell 1976). By contrast, in animals and microorganisms, a clear function of glutathione is best demonstrated when they are exposed to drugs, pesticides or peroxide-generating chemicals (Ziegler 1985).

*Abbreviations* – DHA, dehydroascorbate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; TNB, 2-nitro-5-thiobenzoic acid.

## In vitro characterization of glutathione reductase (EC 1.6.4.2)

### Assay

The standard assay for GR is to follow spectrophotometrically the decrease in absorbance at 340 nm as NADPH is oxidized (Tietz 1969, Carlberg and Mannervick 1985). This assay is rapid, sensitive and accurate, and suitable for characterizing the purified enzyme. However, it is less reliable for assaying activity in plant tissue homogenates, because of the presence of other NADPH oxidizing enzymes and high background absorbance at 340 nm.

These latter considerations led us to develop a more specific assay based on the increase in absorbance at 412 nm when 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is

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Tab. 1. Selected characteristics of plant glutathione reductases.

| Plant source | Purification fold | Molecular, native | Weight subunit | $K_m$ ($\mu M$) | | Literature source |
|---|---|---|---|---|---|---|
| | | | | GSSG | NADPH | |
| Corn (Chloroplast) | 200 | 190 | 65, 63, 34, 32 | 14 | 1.7 | Mahan and Burke, 1987 |
| Pea (Chloroplast) | 619 | | 60, 32 | 62 | 3 | Connell and Mullet, 1986 |
| Pea (Chloroplast) | 1600 | 156 | 41, 42 | 11 | 1.7 | Kalt-Torres et al., 1984 |
| Pea (Chloroplast) | 1000 | 135 | | 28 | 2.7 | Bielawski and Joy, 1986b |
| Pea root | 2400 | 135 | | 10 | 2.3 | |
| Spinach (non-hardened) | Assayed at 25°C | | | 38 | 8 | Guy and Carter, 1984 |
| | Assayed at 5°C | | | 24 | 8 | |
| Spinach (hardened) | Assayed at 25°C | | | 52 | 6 | |
| | Assayed at 5°C | | | 5 | 2 | |

reduced by GSH to produce 2-nitro-5-thiobenzoic acid (TNB) (Tietz 1969, Smith et al. 1988), according to the following equations:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad (1)$$

$$GSH + DTNB \longrightarrow GS\text{-}TNB + TNB \quad (2)$$

$$GSH + GS\text{-}TNB \longrightarrow GSSG + TNB \quad (3)$$

$$GS\text{-}TNB + NADPH + H^+ \xrightarrow{GR} GSH + TNB + NADP^+ \quad (4)$$

Although reactions (1) and (2) are critical to the assay, GS-TNB is an excellent substrate for GR, indicating the importance of reaction (4) (Eyer and Podharadsky 1986). This assay should not be used for kinetic characterization of the enzyme, because of the separate contributions of GSSG and GS-TNB, which have different $K_m$, to the overall rate of TNB production. However, when assaying GR in unpurified homogenates of plant tissue, DTNB has several advantages. Firstly, the absorbance of plant extracts is lower at 412 nm than it is at 340 nm, and the absorbance increase at 412 nm, in response to a fixed amount of enzyme, is from 3- to 4-fold greater than the absorbance decrease at 340 nm; secondly, the assay is not significantly influenced by a variety of compounds commonly included in extraction media; and finally, the assay is specific for GR and is less influenced by the presence of other NADPH oxidizing enzymes than is the traditional assay.

Before leaving the subject of GR assays and quantitation of glutathione using yeast GR, we should caution that the tripeptide specificity of GR has not been examined. It is assumed that the GR from yeast utilizes glutathione and homoglutathione equally well, and that glutathione is a suitable substrate for assaying the GR from homoglutathione-containing species. These assumptions should be tested, because it is known that the presence of homoglutathione, glutathione or a combination of these tripeptides in particular species is due to differences in the specificities of the "glutathione synthetases" (Macnicol 1987).

### Purification and properties

Glutathione reductases have been extensively purified from corn, pea, and spinach (Tab. 1). Purification is facilitated by the compartmentation of a significant amount of the leaf activity in the chloroplast, and affinity chromatography using 2',5'-ADP Sepharose. The native molecular weight ranges from 135 to 190 kDa, depending on the source, and the enzyme is a heterotetramer composed of subunits of approximately 60 and 32 kDa. Reports of a lower molecular weight (Kalt-Torres et al. 1984) are thought to result from partial proteolytic digestion of the native enzyme (Connell and Mullet 1986). The smaller subunit lacks catalytic activity, but its function, particularly its role in regulating catalytic activity is unknown (Connell and Mullet 1986).

NADPH is the preferred reductant for plant GR, a property shared with enzymes from animal and microbial sources. The $K_m$ for GSSG and NADPH are in the ranges 10–60 and 2–10 $\mu M$, respectively. The physiological significance of these numbers is that they are more than an order of magnitude lower than the concentration of these compounds in the chloroplast. Specifically, the total concentration of glutathione in chloroplasts is between 1 and 4 m$M$, 10% of which is GSSG (Bielawski and Joy 1986a); this is probably an underestimate, because chloroplasts prepared by aqueous procedures contain a lower fraction of the total leaf glutathione than do chloroplasts prepared by non-aqueous methods (Smith et al. 1985, Klapheck et al. 1987). The concen-

tration of NADPH is more than 0.1 m*M*, although a relatively small fraction of this amount may be available for GSSG reduction.

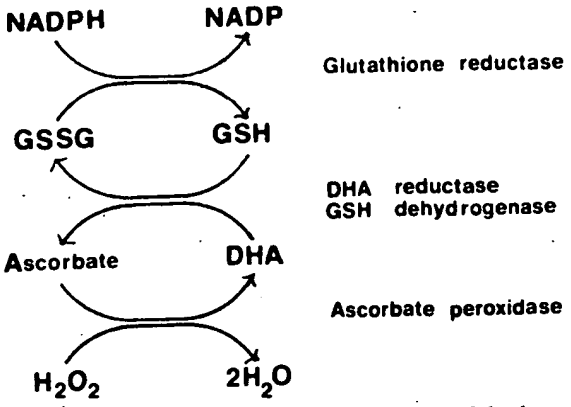
Chloroplasts have been the source of enzyme in most plant studies. However, Bielawski and Joy (1986b) demonstrated the presence in pea roots of an isozyme that differed from the chloroplastic GR: the root enzyme had a higher affinity for GSSG and NADPH, was more sensitive to inhibition by $Zn^{2+}$, $Cu^{2+}$ and $Fe^{2+}$ ions, and slightly more resistant to heat inactivation. Similarly, multiple forms of GR occurred in spinach. When plants were grown at 5°C (hardened), two isozymes appeared that were not present in non-hardened plants, and the relative abundance of isozymes common to both kinds of plants changed (Guy and Carter 1984). The physiological significance of these changes is suggested by the increased affinity of the total pool of GR for GSSG and NADPH, when assayed at low temperature (Tab. 1).

GR undergoes a redox interconversion when incubated with substrates. Incubation of the enzyme with GSSG produces an oxidized form of the enzyme, which is more stable than the reduced form and not inhibited by N-ethylmaleimide or divalent metal ions, such as $Zn^{2+}$, $Cu^{2+}$ and $Fe^{2+}$ (Halliwell and Foyer 1978, Kalt-Torres et al. 1984, Bielawski and Joy 1986b, Mahan and Burke 1987). Reduction of the enzyme with NADPH causes irreversible inactivation (Kalt-Torres et al. 1984, Bielawski and Joy 1986b), this contrasts with the yeast enzyme, which can be reactivated (Pinto et al. 1985).

The detailed characterization of GR from pea and spinach chloroplasts leaves several critical questions unanswered, including: What is the tripeptide specificity of the enzymes from glutathione and homoglutathione-containing species? How do extrachloroplastic and chloroplastic enzymes differ? Which isozymes are most important in plant responses to environmental stress?

### Role of glutathione reductase in $H_2O_2$ detoxification

The participation of GSH and GR in the $H_2O_2$ scavenging pathway in chloroplasts is the best documented role of glutathione in plants. $H_2O_2$ is generated when $O_2$ is photoreduced by the primary electron acceptor of photosystem I (Robinson and Gibbs 1982). In 1976, Foyer and Halliwell proposed the following pathway of $H_2O_2$ photoreduction, frequently termed photoscavenging:




The three enzymes, glutathione reductase, dehydroascorbate reductase (glutathione dehydrogenase) and ascorbate peroxidase, catalyze reactions that maintain large pools of GSH and ascorbate in the chloroplast and channel reducing equivalents from NADPH to $H_2O_2$.

Several lines of evidence support the operation of this pathway. First, intact chloroplasts photoreduce $H_2O_2$ at rapid rates with the concomitant evolution of $O_2$ (Nakano and Asada 1981, Jablonski and Anderson 1982, Anderson et al. 1983a). This ability is partially or totally lost when they are preincubated with $H_2O_2$ in the dark, because of the loss of ascorbate and the concomitant inactivation of ascorbate peroxidase (Anderson et al. 1983b, Asada and Badger 1984, Hossain and Asada 1984a, Nakano and Asada 1987). Secondly, ruptured cholorplasts have very low photoscavenging activity, but recover the ability to photoreduce $H_2O_2$ when both GSH and DHA are added (Nakano and Asada 1981, Jablonski and Anderson 1982). The partial reactions of the system have also been demonstrated, for instance, reconstituted chloroplast systems support GSSG-dependent $O_2$ evolution when NADPH is provided, but intact chloroplasts do not, because the chloroplast membrane is impermeable to GSSG (Jablonski and Anderson 1978, Nakano and Asada 1981, Anderson et al. 1983b). Third, all of the necessary enzymes have been isolated, purified and shown to occur in chloroplasts (Foyer and Halliwell 1977, Nakano and Asada 1981, Jablonski and Anderson 1982, Anderson et al. 1983b, Hossain and Asada 1984b, Bielawski and Joy 1986a, Gillham and Dodge 1986, Nakano and Asada 1987). Jablonski and Anderson (1981) concluded that GR is the rate limiting enzyme in the $H_2O_2$ scavenging pathway.

The physiological importance of the $H_2O_2$ scavenging system is suggested by two recent studies. In field-grown spruce (*Picea abies* L.), there is a two-fold diurnal change in the concentration of glutathione, that is correlated with light intensity (Schupp and Rennenberg 1988). Although glutathione concentration in pea chloroplasts is not influenced by light intensity, the levels of GR, ascorbate peroxidase and DHA reductase increase 50 to 100% in response to increases in light intensity (Gillham and Dodge 1987). Further, the elevated levels

of these enzymes at the higher light intensity reduce the rate of paraquat-induced chlorophyll bleaching.

In summary, a solid body of evidence confirms the operation of the Foyer-Halliwell $H_2O_2$ scavenging system in chloroplasts, and calculations based on enzymatic rates and substrate concentrations support the conclusion that it can operate at the rates necessary to detoxify the $H_2O_2$ generated in the light by the Mehler reaction.

### $H_2O_2$ reduction outside of the chloroplast

The enzymes discussed in the previous section are located primarily in the chloroplast, but sufficient activity is present in the cytoplasm to allow the operation of a similar pathway there, which is the case in *Euglena*, where the enzymes are exclusively extrachloroplastic (Shigeoka et al. 1987). In legumes, all three enzymes are present in the root nodules (Dalton et al. 1986, 1987); in peas, the GRs purified from chloroplasts and roots are different (Bielawski and Joy 1986b).

$H_2O_2$ generated during photorespiration, through the action of glycolate oxidase, is normally degraded by a peroxisomal catalase (Tolbert 1981). In a catalase-deficient mutant of barley and when barley, soybean and tobacco are treated with the catalase inhibitor, aminotriazole, photorespiratory $H_2O_2$ causes a 5 to 10-fold accumulation of glutathione, primarily as GSSG. (Smith et al. 1984, Smith 1985). Transfer of plants to 2.5% $CO_2$, in the light or dark, results in the reduction of GSSG to GSH (Smith 1985; I. K. Smith, unpublished results).

The mechanism whereby $H_2O_2$ causes oxidation of extrachloroplastic GSH is controversial. In animals, $H_2O_2$ and various organic peroxides in the cytoplasm are reduced by a selenium-dependent glutathione peroxidase (EC 1.11.1.9) (Meister and Anderson 1983), but Smith and Shrift (1979) could not demonstrate this activity in a variety of plants and microorganisms. The issue has been further clouded by recent work with algae. Yokota et al. (1988) reported that addition of sodium selenate to the culture medium of the green alga, *Chlamydomonas reinhardtii*, caused the disappearance of ascorbate peroxidase and the appearance of glutathione peroxidase. By contrast, Gennity et al. (1985) reported that selenate-induced peroxidation of glutathione in the green alga, *Dunalliella primolecta*, and the red alga, *Porphyridium cruentum*, was non-enzymatic and they suggested that glutathione peroxidation in the absence of selenate could be due to the operation of a "Foyer-Halliwell" cycle. These studies emphasize the need to purify cell homogenates before drawing conclusions about the enzymes that facilitate cell processes. Less easy to discount is the work of Overbaugh and Fall (1985). They purified to homogeneity a glutathione peroxidase from *Euglena gracilis*, which lacked Se, and had a relatively high affinity for GSH (0.7 m*M*) and $H_2O_2$ (0.03 m*M*). On the other hand, Jablonski and Anderson (1984) clearly showed that $H_2O_2$-dependent oxidation of glutathione by crude extracts from pea shoots requires a flavonoid, tentatively identified as kaempferol-3-(*p*-coumaroytriglucoside) (KCG). They suggested that more than one protein was involved, with one being a peroxidase belonging to the EC 1.11.1.7 class, as follows:

$$\text{KCG} + H_2O_2 \longrightarrow \text{phenolic oxidation product} + H_2O$$
$$\text{oxidized phenol} + 2\,\text{GSH} \longrightarrow \text{KCG} + \text{GSSG}$$

$$\text{Net} \quad 2\,\text{GSH} + H_2O_2 \longrightarrow \text{GSSG} + 2H_2O$$

In conclusion, whether extrachloroplastic $H_2O_2$ oxidizes GSH directly, via a Se-dependent or Se-independent glutathione peroxidase, or indirectly, via the Foyer-Halliwell cycle or an oxidized phenol, GR will be required to regenerate GSH.

### Thiol:disulfide status and biological stress

The idea that glutathione and GR are involved in tolerance to or avoidance of biological stress was developed in Levitt's thiol:disulfide hypothesis of frost injury and resistance in plants (Levitt 1962). According to this hypothesis, frost resistance would result from prevention of thiol oxidation, thiol:disulfide interchange and formation of intermolecular disulfides. Since 1962, the recognized importance of lipids as the major structural component of membranes has resulted in more emphasis on lipid oxidation, but even here, GSH may play a key role in protecting membranes from free radical damage by trapping oxygen radicals in the aqueous phase (Barclay 1988). Discussed solely in the context of glutathione, the following are required; a pool of glutathione for the oxidant scavenging or reducing system, an active GR to regenerate GSH, a supply of NADPH, and operation of the system at low temperature. The latter is a crucial, but frequently overlooked consideration.

### Low temperature stress

Evidence that glutathione and GR play a role in frost tolerance is correlative. The paper by Esterbauer and Grill (1978) on seasonal variation of glutathione and GR in needles of spruce is typical and often quoted. They measured an annual cycle during which glutathione increased from 70 nmol (g fresh weight)$^{-1}$ in August to 700 nmol (g fresh weight)$^{-1}$ in February and March, and then declined. Similarly, the average extractable GR activity in the leaves of 22 evergreen winter hardy plants varied from 0.76 units in July to 2.58 units in February. At least in spruce, these yearly cycles were independent of the age of the needles and continued for several years of the study.

However, the work of Guy, Carter and coworkers (1982, 1984) illustrates the danger of correlations. In

Tab. 2. Effect of l

| Plant |
|---|
| Dogwood |
| Citrus |
| Spinach |
| Soybean primary leaves |
| Soybean second trifoliate |

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Tab. 2. Effect of low temperature on glutathione and glutathione reductase levels in selected plants.

| Plant | Conditions, weeks at °C | | GR, U (g FW)$^{-1}$ | GSH nmol (g FW)$^{-1}$ | GSSG | GSH / GSSG | Literature source |
|---|---|---|---|---|---|---|---|
| Dogwood | 4 | 25/20 | | 90 | 43 | 2.1 | Guy et al., 1984 |
| | 6 | 5/5 | | 590 | 90 | 6.6 | |
| Citrus | 1 | 25/20 | 1.1 | | | 2.0 | |
| | 4 | 10/10 | 2.0 | | | 11.5 | |
| Spinach | 1 | 5/5 | 1.0 | 140 | 29 | 4.8 | Guy and Carter, 1984 |
| | 2 | | 1.3 | 240 | 27 | 9.0 | |
| | 3 | 5/5 | – | 320 | 20 | 16.0 | |
| | 4 | 5/5 | 1.6 | – | – | – | |
| | 4 | 25/20 | 1.0 | 92 | 18 | 5.1 | |
| Soybean primary leaves | 0 | 5/5 | 2.2 | 860 | 10 | 86 | T. L. Vierheller and I. K. Smith (unpubl.) |
| | 1 | 5/5 | 1.8 | 1880 | 230 | 8.2 | |
| | 2 | 5/5 | 1.8 | 1060 | 590 | 1.8 | |
| | 3 | 5/5 | 1.8 | 290 | 1390 | 0.2 | |
| | 0 | 35/25 | 2.2 | 930 | 10 | 93 | |
| | 1 | 35/25 | 1.8 | 470 | 10 | 47 | |
| | 2 | 35/25 | 1.7 | 390 | 10 | 39 | |
| | 3 | 35/25 | – | 700 | 10 | 70 | |
| Soybean second trifoliate | 0 | 5/5 | 2.1 | 1090 | 20 | 55 | T. L. Vierheller and I. K. Smith (unpubl.) |
| | 1 | 5/5 | 2.2 | 2690 | 160 | 17 | |
| | 2 | 5/5 | 1.7 | 2450 | 760 | 3.2 | |
| | 3 | 5/5 | 1.7 | 2200 | 2190 | 1.0 | |
| | 0 | 35/25 | 1.8 | 940 | 20 | 94 | |
| | 1 | 35/25 | 2.0 | 870 | 10 | 87 | |
| | 2 | 35/25 | 1.5 | 680 | 10 | 68 | |
| | 3 | 35/25 | 1.4 | – | – | | |

1982 they demonstrated a correlation between glutathione content and frost hardiness, by comparing potato leaves, ivy leaves and dogwood stems. They implied, without explicitly stating, that a causal relationship existed. However, in 1984 they concluded that GSH content does not have to increase for cold acclimation in dogwood. Exposure of plants to 5°C for 6 weeks in a growth chamber resulted in increases in GSH (550%) and GSSG (109%) (Tab. 2). However, when plants were grown outside, glutathione did not accumulate in the fall, even though the freezing tolerance of the stems increased dramatically. Disagreement between results obtained in growth chamber studies and field studies suggest that interactions between photoperiod, temperature and light may be more important in determining the plant's response to low temperature than any single parameter. In orange seedlings (*Citrus sinensis* L. Osbeck), GSH, GR and the GSH:GSSG ratio increased during cold acclimation at 10°C. However, treatment of plants with dichlormid, a herbicide antidote that increases the glutathione content, does not increase their freezing tolerance, again leading the authors to conclude that neither glutathione content, glutathione reductase activity nor GSH to GSSG ratio has any direct involvement in the development of leaf hardiness in citrus. (Guy et al. 1984). They suggested that the accumulation of glutathione – in plants exposed to low temperature with adequate sulfate – was due to the storage of excess reduced sulfur when the demand for sulfur-containing metabolites declined (see Rennenberg 1982, 1984). Similarly, de Kok et al. (1981) found no significant change in frost tolerance of spinach leaves, as measured by electrolyte leakage, when the SH content was raised from 50 to 250 nmol (leaf disc)$^{-1}$. de Kok and Oosterhuis (1983) demonstrated a two-fold increase in water soluble non-protein SH and GR activity in spinach exposed to short days (8 h) and low temperature (4°C), and the frost tolerance of the plants was lowered by more than 4°C. However, shoot growth was reduced more than 80% at the lower temperature, indicating that a variety of metabolic and developmental processes were affected. When soybean plants are grown at 5°C, the total amount of glutathione increases along with a significant increase in the percentage of GSSG. In addition, trifoliate leaves have a greater increase in total glutathione than primary leaves. The amount of extractable GR is not influenced by growth at 5°C (Tab. 2; T. L. Vierheller and I. K. Smith, unpublished results).

Many enzymes are adapted to function optimally within the normal temperature range of the organism: kinetic parameters that are highly temperature sensitive are $K_m$ and $K_{cat}$ (Somero 1978). Burke and coworkers

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(1987) utilized the concept of a "thermal kinetic window", which is the optimal thermal range for an organism. The apparent $K_m$ of a critical enzyme would have its minimal value and maximal insensitivity to temperature in this range (Burke et al. 1987, Mahan et al. 1987). The GR from several species have $K_m$s that vary 6-fold between 15 and 45°C (Mahan et al. 1987). The "thermal kinetic window" was calculated to be from 12.5 to 22°C in spinach. However, in a previous study, the lower $K_m$s for cold-hardened spinach was attributed to accumulation of a new isozyme (Guy and Carter 1984). These results highlight the danger of measuring $K_m$s in unfractionated cell homogenates: it is impossible to know whether the $K_m$ of the predominant isozyme is being influenced by environmental conditions or if the change in "composite $K_m$" is due to a change in the relative amounts of isozyme with different $K_m$s.

### High temperature stress

Studies of the effect of high temperature stress on glutathione levels and extractable GR are limited. In maize (*Zea mays* L.), heat shocking of roots at 40°C caused an increase (17%) in glutathione by increasing the activity of the biosynthetic enzymes (Nieto-Sotelo and Ho 1986). In the same study, however, an unidentified compound, which was not a thiol, increased 8-fold indicating that the change in glutathione was modest. Recently, GSH was shown to stimulate transcription of various defense genes when supplied to suspension cultured cells of bean (*Phaeolus vulgaris* L.; Dron et al. 1988, Wingate et al. 1988). Among the genes affected were those that encode cell wall hydroxyproline-rich glycoprotein, phenylalanine ammonia lyase and chalcone synthase. Two aspects of this study are notable; first, the response was elicited by very low (0.01 m*M*) concentrations of GSH, considering that the concentration of GSH in the cytoplasm is probably higher than 0.1 m*M* (Klapheck et al. 1987) and some of the transported GSH is degraded (Rennenberg 1981); second, the response was not elicited by GSSG, which would be a more reliable indicator of stress than a modest increase in GSH, considering that GSH normally varies 2-fold during the day (Schupp and Rennenberg 1988). This study raises important questions that merit further investigation.

### Oxidative and water stress

The potential role of GR and glutathione in tolerance mechanisms to oxidative and water stresses has been examined in several plants, but the results are equivocal.

Exposure of maize and cotton (*Gossypium hirsutum* L.) plants to an atmosphere containing 75% $O_2$ resulted in a 2- to 3-fold increase in extractable glutathione reductase (Foster and Hess 1980, 1982). The GR activity was twice as high in two ozone-insensitive cultivars of bean (*Phaseolus vulgaris* L.) when compared to two ozone-sensitive cultivars, although enzyme activity was not influenced by $O_3$ fumigation (Guri 1983). By contrast, extractable enzyme activity was similar in ozone-sensitive and ozone-insensitive cultivars of spinach fumigated with ozone for 1 day (Tanaka et al. 1985). However, the amount of GR, measured immunologically, increased in an ozone-sensitive cultivar exposed to ozone for several days (Tanaka et al. 1988).

Drought influences the amount of GR activity present in leaves, but the means whereby the enzyme is elevated relative to controls depends on the plant. In a glasshouse study with barley, withholding water for 5 days caused an increase in GR (Smirnoff and Colombe 1988). By contrast, water stress induced increases in enzyme activity in field grown cotton and winter wheat (*Triticum aestivum* L.) were usually due to inhibition of the decline in activity, which normally occurs over a growing season in irrigated plants (Gamble and Burke 1984, Burke et al. 1985). These latter studies also showed that leaf position and planting density are important variables when investigating the effect of water stress on GR levels. Another dependent variable in field studies is temperature, because canopy temperatures of irrigated plants are on average lower than those of dryland plants (Burke and Hatfield 1987).

### Concluding remarks

The role of glutathione and GR in the $H_2O_2$-scavenging pathway in chloroplasts is well established. By contrast, the functions of extrachloroplastic GR, under normal or stressed conditions, have received less attention; despite this, the available literature suggests some guidelines for studies aimed at defining additional functions of GR. At the least, the following should be considered; changes in the total pool of GR may be less significant than changes in individual isozymes; in stress phenomena, investigations using resistant, tolerant and sensitive genera may be more illuminating than the effect of stress on a single genus or species; and finally, results obtained in greenhouse experiments must be confirmed in field studies, before conclusions can be drawn regarding the adaptive significance of the results.

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# CORRELATION BETWEEN CuZn SUPEROXIDE DISMUTASE AND GLUTATHIONE REDUCTASE, AND ENVIRONMENTAL AND XENOBIOTIC STRESS TOLERANCE IN MAIZE INBREDS

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A hypothesis was tested that there would be cross-tolerance between various oxidant stresses, using drought-tolerant and intolerant maize inbreds. Membrane leakage was measured to determine oxidant damage without needing quantification of the enzymes and other factors involved in protection. Paraquat and acifluorfen tolerances paralleled drought and $SO_2$ tolerances using this test. Thus, membrane leakage caused by oxidant generating herbicides can also be used to predict drought tolerance; a much harder character to ascertain. Drought and photooxidative herbicide tolerances were both significantly correlated with high levels of CuZn superoxide dismutase (EC 1.15.1.1.) and with glutathione reductase (EC 1.6.4.2) activities. High levels of just superoxide dismutase or just glutathione reductase did not correlate with any of the tolerances.

*Key words*: CuZn superoxide dismutase; glutathione reductase; membrane leakage; paraquat resistance; stress cross tolerances; *Zea mays* L.

## Introduction

The antioxidant systems in plants act as important stress tolerance mechanisms by protecting membranes against damage caused by the toxic oxygen species; superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($^{\cdot}OH$) produced under environmental and xenobiotic stress conditions [1–6].

Cross tolerances were found between oxidant generating herbicides and environmental oxidants in different plant biotypes constitutively tolerant to paraquat, $SO_2$, $O_3$ [6], and to photoinhibition [7]. They also correlated high levels of the Halliwell-Asada oxygen detoxifying pathway with tolerance to the various oxidants as determined biochemically [5], and by genetic experiments [8]. Indeed, enzymatic activity of the Halliwell- Asada active oxygen detoxification pathway [3,4] has been separately correlated with induced resistance to moisture stress [1,9], hyperoxia stress [10], photo-oxidative plant tissue destruction by the herbicide paraquat [11], salinity [12], and $O_3$ stress [13]. Although it seems logical, there had been no previous correlation between constitutively high levels of these enzymes and transient drought tolerance.

Paraquat radical is formed by siphoning electrons from photosystem I. This radical is rapidly reoxidized by molecular oxygen resulting in the formation of superoxide radical anion ($O_2^-$) and paraquat. Superoxide can be a potent oxidant by itself or after dismutation to $H_2O_2$ and formation of hydroxyl radicals ($^{\cdot}OH$) in a Haber-Weiss reaction, or singlet oxygen ($O_2^1$) after reduction [5,14,15]. Similarly, nitrodiphenylether herbicides such as acifluorfen generate active oxygen species by stimulating the accumulation of protoporphyrin IX, a photodynamic pigment [16]. These active oxygen species are all very reactive and cause a cascade of lipoxidations resulting in chlorophyll bleaching and breakdown of plastid and cytoplasmic membranes [5,14]. Leakage of electrolytes [6,17], chlorophyll bleaching [6], and loss of photosynthetic activity [1,5,14] are symptoms of active oxygen reactions with these membranes and chlorophyll.

The measurement of antioxidant activity by

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the quantification of the activities of the Halliwell-Asada pathway enzymes (superoxide dismutase, ascorbate peroxidase and glutathione reductase) [3,4] as well as their substrates is difficult, time consuming and expensive. Other measurements have been used; lipoxidation mediated formation of malondialdehyde [18], ethane evolution from oxidized tissue [19], and loss of chlorophyll content [6]. It is not practical to use the above techniques for screening large amounts of genetic material for possible stress resistance, as an extension of such correlations.

Potassium efflux can be measured using flame photometry, a potassium-specific electrode or by atomic absorption spectrophotometry. Conductivity is an easy and inexpensive technique to measure total electrolyte leakage [17]. We demonstrate below that conductivity correlated with other measurements of stress tolerance in vivo and at the enzyme level.

We present evidence supporting the hypothesis that constitutive cross-tolerance exists between chemical and environmental oxidant stresses using known drought tolerant and intolerant maize inbreds. Plants with a high potential for antioxidant activity should be protected against toxic oxygen species formed during oxidative stress and we show that they have little or no membrane damage when stressed. We also present data to support the hypothesis that plants selected for stress tolerance by the above-mentioned technique, are cross-tolerant to various other oxidant stresses.

## Materials and Methods

### *Plant material*

Thirteen maize (*Zea mays* L.) inbreds commonly used as breeding material in South Africa, including four with known tolerance characteristics (Table I), were planted in 25-l pots filled with quartz sand and irrigated with a nutrient solution, as previously described [20]. Four plants per pot with 4 replicates were planted in the glasshouse and kept at 30 °C day/18 °C night ± 2 °C and 12h photoperiod. Samples for all tests were taken from the youngest fully expanded leaf at the time of sampling. The known tolerance characteristics (Table I) are based on visual pre-flowering reaction under field stress conditions and possible interval changes, due to stress, between anthesis and silking were eliminated by hand pollination.

As no isogenic lines for the Halliwell-Asada pathway exist, two genetic lines each of the extremes in ranking for paraquat tolerance/sensitivity (Table I) were used in cross-tolerance studies.

### *Membrane leakage*

Four replicates, with two eight millimeter diameter leaf disks per replicate, were taken from test plants and incubated for 30 min at 25 °C and 48 $\mu$E $m^{-2}$ $s^{-1}$ in a growth chamber in paraquat, acifluorfen (sodium), or sodium sulfite in 50 mM MES (pH 7.2) to generate $SO_2$ as per Mikkelson et al. [21]. All solutions contained 1% Agrowett (Agroserve) or 0.2% Tween 20 to ensure even and complete wetting of leaf disks.

After a 30-min incubation in the different solutions, the leaf disks were washed and then incubated for 24 h in 10 ml deionized water in test tubes at room temperature in the light. The leaf disks were removed and solution was filtered through 0.45 $\mu$m Millipore filters.

### *Potassium efflux*

Each solution was spiked to a final concentration of 1 g $l^{-1}$ cesium chloride to suppress ionization of potassium in the flame of the atomic absorption spectrophotometer was calibrated with 1–8 mg $l^{-1}$, $K^+$ and the potassium efflux resulting from the paraquat-induced membrane damage of cells was determined for each solution.

### *Conductivity*

Total electrolyte leakage was measured in the cross-tolerance studies, employing a Radiometer type CDM2e conductivity meter after treatment of leaf disks with acifluorfen or $SO_2$.

*Drought treatment*

Six selected maize inbreds were planted as before and watering was stopped on day 30 after planting. Relative water content determinations [21] were performed at regular intervals for each of the six inbreds until a defined maximum moisture stress of approximately 55% relative water content was reached. Leaf material of each inbred was collected at all intervals, freeze dried and ground in a water cooled grinder. This ground, dry leaf material was used to determine Mn and CuZn superoxide dismutase activities as well as glutathione reductase activity. Additional data were collected at regular intervals on $K^+$-leakage, as well as malondialdehyde accumulation measured spectrophotometrically at 535 nm after reaction with thiobarbituric acid [17]. Chlorophyll was extracted, measured spectrophotometrically at 645 and 663 nm, and the concentration determined according to Arnon [23].

*Enzyme analyses*

Two hundred milligrams of ground leaf material were extracted in 5 ml 0.1 M potassium phosphate extraction buffer (pH 7.5) containing 0.1 mM EDTA, 200 mg polyvinylpyrrolidone (mol. wt. = 25 000), 1% w/v bovine serum albumin and 200 $\mu$M $\beta$-mercaptoethanol and filtered through Whatman GF/A glass fiber filters. The extracts were used to determine glutathione reductase and superoxide dismutase activities. The level of glutathione reductase activity was measured using the technique described by Carlberg and Mannervik [24] by following the oxidation of NADPH spectrophotometrically at 340 nm. The initial rate of the reaction was determined and activity was expressed as $\mu$mol of NADPH oxidized $min^{-1}$ 0.2 $g^{-1}$ dry weight sample. Mahan and Burke [25] claimed that 5 mM $\beta$-mercaptoethanol inhibits glutathione reductase activity. Other workers [5,6,8] included $\beta$-mercaptoethanol in extraction buffers and were able to measure glutathione reductase activity, as is also shown in this study. The levels of Mn and CuZn superoxide dismutase activities were also measured using the above extracts according to Geller and Winge [26]. This method involves the inactivation of Mn superoxide dismutase by sodium dodecyl sulfate (SDS), which does not affect CuZn superoxide dismutase. The SDS was then removed by precipitation with 3 M KCl at 6°C and centrifugation at 20 000 × $g$. The supernatant was assayed for superoxide dismutase activity, measuring inhibition of nitrite formation from hydroxylammonium chloride oxidation at 530nm as described by Elstner and Heupel [27]. Mn superoxide dismutase activity was measured by assaying the sample before SDS treatment in the presence of 2 mM cyanide, which inhibits the CuZn enzyme by 94% [26] but does not affect the Mn enzyme. Units (U) of activity were calculated from absorbance values obtained by treating a known concentration range of superoxide dismutase from horseradish (Sigma) as described above. A unit of activity for this superoxide dismutase is defined as that amount which causes 50% inhibition of cytochrome c reduction as per McCord and Fridovich [28].

## Results and Discussion

*Paraquat Effects*

Increasing paraquat concentration should result in an increase of active oxygen in the plant tissue and thus cause an increase membrane leakage. These increases in membrane damage were clearly demonstrated by the increase in $K^+$-efflux and total electrolyte leakage (Figs. 1 and 2, respectively). There is a noticeable loss in sensitivity to paraquat as test plants get older (Fig. 1), even though leaves of the same developmental stage were used for all determinations. The antioxidant system might function more effectively in older plants, as was shown for glutathione reductase activity in upper leaves of wheat [29]. However, the opposite was found for upper leaves of cotton [1], and Tanaka and Sugahara [30] found that young poplar leaves had five times more superoxide dismutase than the older leaves, and the younger leaves were more resistant to $SO_2$.

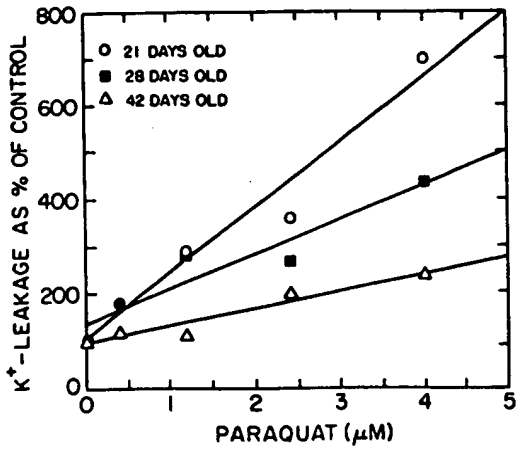


Fig. 1. The effect of paraquat on $K^+$-efflux from maize leaves of similar development but on different aged plants. $K^+$-efflux was measured as percent of untreated controls of maize cultivar P473 leaf discs from the youngest mature leaves sampled from different age plants and treated with paraquat and following the protocol as described in Material and Methods. The untreated control value was 0.5 mg $l^{-1}$ $K^+$.

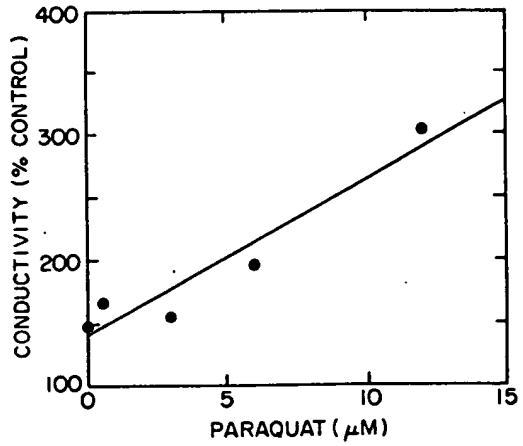


Fig. 2. The effect of paraquat on total electrolyte leakage from maize leaves of 20-day-old plants of inbred 8 (paraquat-sensitive) Total electrolyte leakage measured as conductivity for leaf discs taken from the youngest mature leaves. Leaf discs were incubated in different paraquat concentrations for an hour at 45 $\mu E\ m^{-2}s^{-1}$, washed and left for 24 h in double distilled $H_2O$, whereafter conductivity of the solution was measured and calculated as percent of untreated control. The untreated control value was 150 $\mu$Mho.

*Testing inbreds for paraquat tolerance*

Thirteen maize inbreds were tested for antioxidant activity employing the $K^+$-efflux test to assess the sensitivity of inbreds to paraquat (Table I). Three times more potassium leaked from heat sensitive inbred 2, and 135% more from drought sensitive inbred 3 than from the most paraquat-resistant inbred line 11. Drought-resistant inbred 10, and heat resistant inbred 1 were not quite as resistant to membrane damage as inbred 11. These results already suggest cross tolerance between paraquat-tolerant and environmental stress-tolerant biotypes. Inbreds 11 and 12, were chosen for use in drought experiments as representing the tolerant biotypes, 5 and 7 the intermediate, and 3 and 8, the sensitive biotypes. The tolerant and sensitive pairs were used in cross-tolerance studies.

*Enzyme activities correlated with drought and paraquat tolerance*

Inbreds 11 and 12 (paraquat tolerant) had significantly higher CuZn superoxide dismutase and glutathione reductase activities than the rest of the inbreds, which did not differ significantly from each other (Table I). However, no relationship was found between the Mn superoxide dismutase activity and paraquat susceptibility or tolerance. This is probably due to mitochondrial localization of this enzyme [10,11,31]. Mn superoxide dismutase would not be able to participate in the plastid associated Halliwell-Asada pathway. Matters and Scandalios [10] similarly found that maize plants subjected to elevated $O_2$ stress had increased CuZn superoxide dismutase, but had normal levels of the mitochondrial Mn superoxide dismutase activity. CuZn superoxide dismutase and glutathione reductase are found in the plastids [1,5,29], and a different CuZn superoxide dismutase isozyme is also found in the cytoplasm [5,8,10].

The CuZn superoxide dismutase activity of inbred 8 and glutathione reductase activity of inbred 3 (both paraquat sensitive) were the lowest (Table I). This may indicate that the activities of different enzymes could be rate

Table I. Paraquat tolerance and oxidant detoxifying enzyme activities of 13 maize inbreds.

| Inbreds | Breeders classification[a] | Paraquat tolerance $K^+$-leakage (mg $l^{-1}$)[b] | Antioxidant-enzyme activities[b] | | |
|---|---|---|---|---|---|
| | | | MnSOD (I.U.) | CuZn SOD (I.U.) | Glutathione reductase |
| 11 | | 2.0 a | 3.3 ab | 7.1 a | 24 a |
| 12 | | 2.2 a | 2.9 bc | 7.7 a | 20 b |
| 6 | | 2.2 a | | | |
| 10 | Drought R | 2.7 ab | | | |
| 7 | | 2.8 ab | 2.5 c | 5.1 b | 14 cd |
| 5 | | 3.3 bc | 2.9 bc | 5.2 b | 15 c |
| 1 | Heat R | 3.3 bc | | | |
| 9 | | 3.5 bc | | | |
| 4 | | 4.3 cd | | | |
| 3 | Drought S | 4.7 de | 3.5 a | 5.8 b | 10 d |
| 13 | | 4.8 de | | | |
| 8 | | 5.5 ef | 2.9 bc | 4.9 b | 14 cd |
| 2 | Heat S | 6.2 f | | | |

[a]Where no classification is given, there was no preliminary information.
[b]Values in a column followed by the same letter do not differ significantly at $P \geq 0.05$ according to Duncan's multiple range test. Maize inbreds ranked in order of high to low antioxidant activity, measured as the amount of $K^+$-leakage from 4 μM paraquat treated leaf disks of 20-day-old plants. R, resistant; S, susceptible; SOD, superoxide dismutase; glutathione reductase is expressed in μmol NADPH oxidized per min per 0.2 g dry weight sample.

limiting in the oxidant detoxification pathway, rendering these two inbreds stress sensitive. The data in Table I, demonstrate that there is a direct relationship between concurrent high levels of both enzyme activities and paraquat tolerance (according to the $K^+$-leakage test). High levels of one enzyme alone was never correlated with oxidant stress tolerance.

Three membrane integrity parameters; $K^+$-efflux in paraquat treated leaf discs and the formation of the lipoxidation product malondialdehyde and chlorophyll bleaching during moisture stress were compared with CuZn superoxide dismutase and glutathione reductase activities in the same material (Fig. 3). All correlations between high concurrent CuZn superoxide dismutase and glutathione reductase activities and membrane integrity parameters were significant. The role of higher active oxygen detoxifying enzyme activities in maintaining membrane and chlorophyll intactness during oxidative stress are clearly seen in the negative slopes of the graphs in Fig. 3. The results in Fig. 3 indicate that there are minimum threshold activities for both CuZn superoxide dismutase ≈ 5 I.U. and glutathione reductase ≈ 12 μmol NADPH oxidized $min^{-1}$ per 0.2 g sample, below which there is no membrane protection during stress, resulting in maximum chlorophyll breakdown (Figs. 3E and 3F), $K^+$-leakage (Figs. 3A and 3B) and membrane lipid peroxidation (Fig. 3C and 3D). Threshold activities for maximum membrane protection were found with optimal enzyme activities of approximately 6.5 I.U. CuZn superoxide dismutase and 17 μmol NADPH oxidized $min^{-1}$ per 0.2g sample of glutathione reductase (Figs. 3A–3F). Above these values protection against toxic oxygen species was not further enhanced.

The high antioxidant activity seems to maintain high chlorophyll content under all oxidant stresses. If chlorophyll breaks down, even during reversible membrane damage, it cannot be restored, resulting in a die-back of damaged leaves, inhibition of whole plant photosynthesis and ultimately lower yields. In plants with an efficient oxygen detoxifying

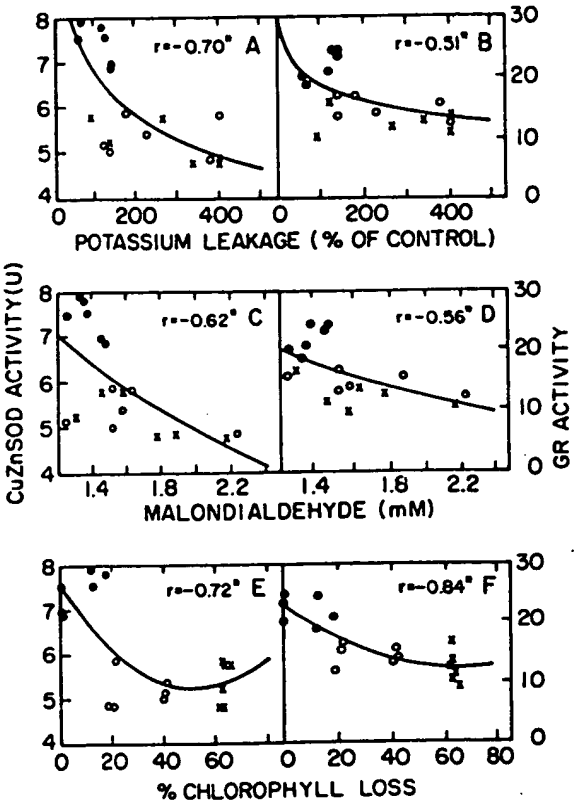


Fig. 3. Correlation between superoxide dismutase and glutathione reductase with membrane integrity parameters. A, B. $K^+$-leakage was measured in paraquat treated plants and C-D malondialdehyde accumulation and E, F chlorophyll content was measured in drought stressed plants (as measures of loss of integrity). Scattergrams are made up from 3 replicate data points for each of the two paraquat/ drought-resistant maize inbreds (inbreds 11, 12), intermediates (inbreds 5, 7) and paraquat-sensitive inbreds (3, 8). The untreated control values over various experiments for membrane integrity parameters are as follows: $K^+$-leakage = 0.5 mg $l^{-1}$, malondialdehyde = 1.3 mM, chlorophyll = 110 $\mu$g $ml^{-1}$. ●, paraquat/drought resistant, O, intermediate, X-paraquat/drought sensitive. Glutathione reductase activity (GR) is expressed as $\mu$mol NADPH oxidized per min per 0.2 g dry weight sample.

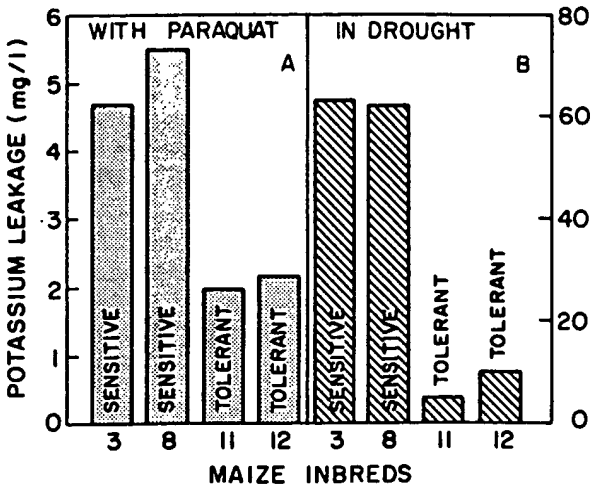


Fig. 4. The prevention of oxidative damage by high oxidant detoxifying enzyme activity levels. A. Membrane integrity of leaf discs treated with 4 $\mu$M paraquat, with damage measured as leakage. Bars show relative oxidant detoxifying activity in drought and paraquat sensitive (3, 8) and drought and paraquat tolerant (inbreds 11, 12). B. Moisture stress tolerance of the same maize inbreds at a relative water content of ≈ 55% K, with damage measured as chlorophyll loss. Untreated control value for $K^+$-leakage is 0.5 mg $l^{-1}$ and 110 $\mu$g $ml^{-1}$ for chlorophyll. The differences between sensitive and tolerant varieties are significant at the $< 0.01$ level, as ascertained by Student's $t$-test.

system to maintain membranes, chloroplasts and chlorophyll are protected, insuring resumption of photosynthesis when the stress is over. Chlorophyll bleaching significantly increased during maximum moisture stress in low antioxidant activity maize inbreds 3 and 8 (Fig. 4B), while membrane damage measured as $K^+$efflux in paraquat treatments increased (Fig. 4A). These results could also suggest that inbreds with high oxidant detoxifying activities, should also be resistant to photoinhibition, as was found for paraquat-resistant *Conyza* [7]. High oxidant detoxifying inbreds should be better able to use light at high fluence rates, allowing more photosynthesis and increased yields.

*Cross tolerance*

Paraquat-tolerant maize inbred 11, paraquat-sensitive inbreds 3 and 8 were used in cross tolerance studies (Table I, Fig. 4). Sensitive inbred 3 was always the most sensitive to paraquat, acifluorfen and $SO_2$ (Fig. 5). Inbred 8 always reacted as an intermediate while inbred 11 was always the most resistant

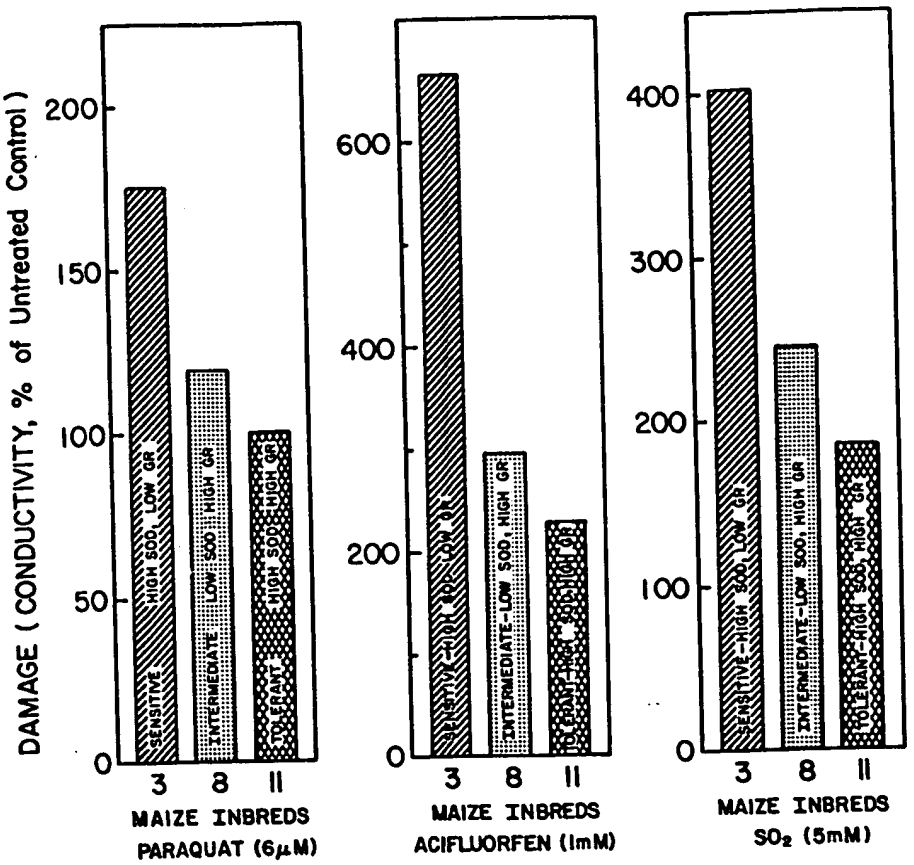


**Fig. 5.** Cross tolerances of 3 maize inbreds to different xenobiotic stresses. Leaf discs of maize inbreds 3, 8 (paraquat-sensitive) and 11 (paraquat-resistant) were treated with different xenobiotics and $SO_2$, and conductivity was measured as a parameter of membrane intactness. The differences between inbred 3 and 11 are significant at the $< 0.01$ level by Student's *t*-test.

by paraquat, acifluorfen, $SO_2$ and drought. The cross tolerances were obvious between the paraquat-tolerant maize inbreds, environmental and other xenobiotic stresses.

The differences in sensitivity to xenobiotic stresses between the two sensitive inbreds are diagrammatically shown in Fig. 6. Drought sensitive inbred 3 has higher CuZn superoxide dismutase activity (Table I) than the minimum threshold of ≈ 5 I.U., but has low glutathione reductase activity (well below the minimum threshold of ≈ 12 μmol NADPH oxidized $min^{-1}$ per 0.2 g sample). This probably results in an accumulation of $H_2O_2$ and a high production of highly potent hydroxyl radicals via the Haber-Weiss reactions, and membranes are damaged (Fig. 6). The inverse of the above situation occurs in maize inbred 8. Inbred 8 has a relatively low CuZn superoxide dismutase activity (beneath the minimum threshold of ≈ 5 I.U., as seen in Table I), but a relatively high glutathione reductase activity (above the minimum threshold of 12 μmol NADPH oxidized $min^{-1}$ per 0.2 g sample). In this case, superoxide, which is not as potent an oxidant as hydroxyl radicals, accumulates and causes less damage (Fig. 6).

Both enzyme activities are well above the minimum threshold activities in the paraquat-tolerant inbred 11, probably resulting in no

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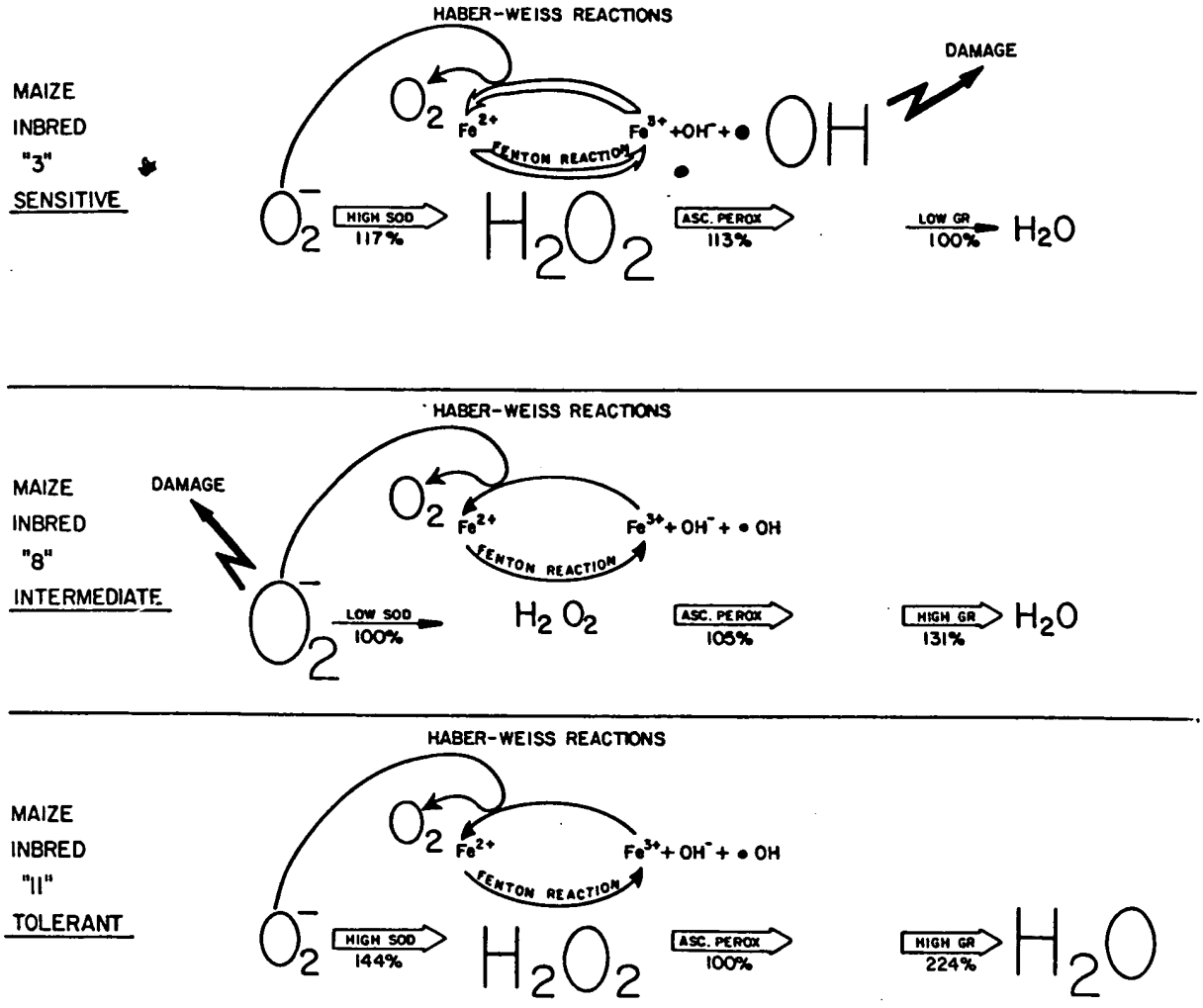


**Fig. 6.** Diagrammatic representation of membrane damage caused in maize inbreds 3 and 8 due to specific enzyme deficiencies with probable explanations for differences in sensitivity to oxidative stress and damage.

accumulation of oxidants or damage (Fig. 6). It is therefore important to note that high levels of just superoxide dismutase or just glutathione reductase are not sufficient to confer tolerance. In maize, definite minimum threshold activities for both enzymes were needed for tolerance. Ascorbate peroxidase, the primary enzyme involved in plastid peroxide detoxification was not measured in this study. Ascorbate peroxidase has been correlated with cross tolerance to oxidant stresses [5,6,8].

The measurement of membrane leakage of paraquat-treated plant material proved to be a rapid and reliable estimate of oxidative stress tolerance as a total integrative function of the antioxidant activity. This happens because stress tolerance correlates well with concurrent high levels of CuZn superoxide dismutase and glutathione reductase activities. Furthermore it was found that biotypes selected for paraquat tolerance by such a technique, had cross tolerance to other xenobiotic stresses

like acifluorfen and $SO_2$, as well as cross tolerance to drought stress. Tolerance to oxidative stress is not increased above an enzyme activity threshold of 6.5 I.U. superoxide dismutase and 17 μmoles NADPH oxidized $min^{-1}$ per 0.2 g sample of glutathione reductase. The fact that chlorophyll content and presumably plastid integrity is preserved in tolerant genotypes, suggests possible photoinhibition tolerance, which could result in more stable and even higher yields in such genotypes in environmental conditions where there is transient drought stress.

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Glutathione reductase

# SUPEROXIDE DISMUTASE AND STRESS TOLERANCE

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KEY WORDS: oxidative stress, plant defense mechanisms, cross-tolerance

## CONTENTS



## INTRODUCTION

Oxidative stress, resulting from the deleterious effects of reduced oxygen species, is an important phenomenon in many biological systems. Superoxide dismutases (SOD) have been identified as an essential component in an

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organism's defense mechanism and have consequently been the subject of much research. In plants, the role of SOD during environmental adversity, has received much attention since reactive oxygen species have been found to be produced during many stress conditions. Here we review this work and discuss the possibilities for generating stress-tolerant plant varieties by the genetic engineering of SOD.

## AN OVERVIEW OF THE DEFENSE MECHANISM AGAINST OXIDATIVE STRESS

The phenomenon of oxidative stress arises from the deleterious reactions of oxygen, which are an unfortunate consequence of life for any aerobic organism. These reactions are mediated by reduced oxygen species such as superoxide radicals and hydrogen peroxide. By themselves they are relatively unreactive, but they can form species damaging to essential cellular components. In the presence of metal ions (such as iron), superoxide and hydrogen peroxide can react in a Haber-Weiss reaction to form hydroxyl radicals:

$$H_2O_2 + O_2^- \xrightarrow{Fe^{2+},\ Fe^{3+}} OH^- + O_2 + OH\cdot \qquad \text{(Haber-Weiss reaction)}$$

Hydroxyl radicals (and their derivatives) are among the most reactive species known to chemistry (reviewed in 28, 77, 79), able to react indiscriminately to cause lipid peroxidation, the denaturation of proteins, and the mutation of DNA. As discussed below, lipid peroxidation is commonly used as an indicator of oxidative stress, although it can be caused by other reactive species (75). In addition, singlet oxygen ($O_2^1$), which is formed when excitation energy is transferred to oxygen, also produces deleterious effects (11, 28, 79, 113).

Superoxide radicals, hydrogen peroxide, and singlet oxygen are formed from many cellular reactions (reviewed in 11, 13, 17, 28, 56, 62, 78, 79, 174). In general, superoxide can arise when electrons are misdirected and donated to oxygen. Mitochondrial electron transport, for example, is a well-documented source of superoxide radicals, as is the electron transport chain of the photosynthetic apparatus within the chloroplasts. An additional problem for chloroplasts is the transfer of excitation energy from chlorophyll to oxygen, which can generate singlet oxygen.

Protective mechanisms have evolved that keep these deleterious reactions to a minimum. Since hydroxyl radicals are far too reactive to be controlled easily, aerobic organisms eliminate the less-reactive forms as efficiently as possible and prevent their coming into contact with each other. This defense



involves both enzymic and non-enzymic mechanisms. Superoxide dismutases (SOD; EC 1.15.1.1), originally discovered by McCord & Fridovich in 1969 (130), react with superoxide radicals at almost diffusion-limited rates to produce hydrogen peroxide:

$$2\ O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(SOD)}$$

This enzyme is unique in that its activity determines the concentrations of $O_2^{\cdot}$ and $H_2O_2$, the two Haber-Weiss reaction substrates, and it is therefore likely to be central in the defense mechanism. Its importance has been established by the demonstration that SOD-deficient mutants of *Escherichia coli* (30) and yeast (21, 215) are hypersensitive to oxygen. It is present in all aerobic organisms (except in rare cases; see 79) and in all subcellular compartments where oxidative stress is likely to arise (see 15, 63).

The three known types of SOD are classified by their metal cofactor: the copper/zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD) forms (for review, see 15). Experimentally, these three different types can be identified by their differential sensitivities to KCN and $H_2O_2$ (e.g. see 105, 214). Cu/ZnSOD is characterized as being sensitive to both $H_2O_2$ and KCN. FeSOD is sensitive to $H_2O_2$ only, and MnSOD is resistant to both inhibitors. The FeSOD and MnSOD enzymes are structurally similar; indeed, the apoenzymes have been found to function with either metal present at the active site (e.g. see 137, 197). The Cu/ZnSOD, however, is structurally unrelated. All prokaryotic organisms so far studied contain MnSOD and/or FeSOD; Cu/ZnSOD is absent except in a few cases (200, and references therein). Eukaryotic algae (except those with phragmoplastic cell division) and protozoa possess MnSOD and FeSOD but not Cu/ZnSOD (7, 8, 104, 118). Cu/ZnSOD has been found in all higher eukaryotes within the animal kingdom, as has MnSOD; while Cu/ZnSOD is cytosolic, MnSOD is found in the mitochondria (15). The phylogenic distribution of SOD thus indicates that MnSOD and FeSOD are ancient; while they probably evolved before eukaryotes and prokaryotes diverged, Cu/ZnSOD has evolved independently at some point near the beginning of the eukaryotic lineage. Hence the enzyme has in fact evolved twice.

Subcellular fractionation studies have been performed in many plant species (e.g. see 15) and in general plants contain a mitochondrial matrix-localized MnSOD and a cytosolic Cu/ZnSOD, with FeSOD and/or Cu/ZnSOD present in the chloroplast stroma. All of the enzymes appear to be nucleus encoded and, where necessary, are transported to their organellar locations by means of $NH_2$-terminal targeting sequences (e.g. 24, 25, 156, 217, 218). The number of isozymes of each type of SOD varies greatly from plant to plant, as does the relative abundance of each enzyme.

Hydrogen peroxide is disposed of by catalases (E.C. 1.11.1.6) and per-

oxidases (EC 1.11.1.7). In plants, catalase is found predominantly in peroxisomes (and also in glyoxysomes) where it functions chiefly to remove the $H_2O_2$ formed during photorespiration (or during $\beta$-oxidation of fatty acids in glyoxysomes) (120, 211). In spite of its restricted location it may play a significant role in defense against oxidative stress since $H_2O_2$ can readily diffuse across membranes. Many different peroxidases occur in plants; unlike catalase, these require a substrate (R) for catalysis:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(catalase)}$$
$$H_2O_2 + RH_2 \rightarrow 2H_2O + R \quad \text{(peroxidase)}$$

Some of these enzymes have a broad substrate specificity while others can only function with one. The peroxidases with broad specificities are often found in the cell wall where they utilize $H_2O_2$ to generate phenoxy compounds that then polymerize to produce components such as lignin (72). In addition to their role in the biosynthesis of cellular components, reactive oxygen species are thought to act as secondary messengers in cells (see below).

Of more importance in the context of oxidative stress is a chloroplast-localized, ascorbate-specific peroxidase activity found mainly in chloroplasts. Together with glutathione reductase and dehydroascorbate reductase it is thought to remove $H_2O_2$ through a mechanism termed the Halliwell-Asada pathway, after its discoverers (11, 61, 78, 145) (Figure 1). Since the action of SOD results in the formation of $H_2O_2$, it is also intimately linked with this pathway. Glutathione reductase, the other key component, has a regulatory function because of the dependence of its activity on the availability of NADPH (reviewed in 193). It has been found not only in chloroplasts but also in mitochondria and cytoplasm (55, and references therein), where it may also cooperate with SOD to remove superoxide radicals. Besides dehydroascorbate, ascorbate peroxidase activity also generates monodehydroascorbate. The ascorbate radical is converted back to ascorbate by a monodehydroascorbate reductase that can use both NADH and NADPH as reductants (22, 91, 92). The importance of this system relative to the Halliwell-Asada pathway remains to be evaluated.

In addition, plant cells contain relatively high levels of ascorbate, glutathione, and $\alpha$-tocopherol, which are efficient oxyradical scavengers. The lipophyllic $\alpha$-tocopherol is present in large amounts in thylakoid membranes where it blocks the chain-propagating reactions of lipid peroxidation (11, 28, 117). Carotenoids are another essential component of thylakoid membranes because they can quench singlet oxygen extremely rapidly (11, 113). Thus SOD is intertwined with other enzymes and antioxidants in what is likely a highly optimized balance that reduces the risk of hydroxyl radical formation. Discussion of the role of SOD therefore necessitates consideration of the oxidant stress defense system as a whole.



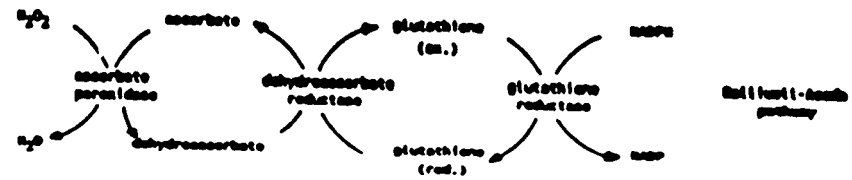

*Figure 1* The Halliwell-Asada pathway.

## RESPONSE OF SOD TO ENVIRONMENTAL STRESS

Chloroplastic SOD is generally the most abundant SOD in green leaves, while in germinating seedlings and in etiolated material the cytoplasmic and mitochondrial SODs are prevalent (60, 100, 103, 105, 169, 177, 213). This distribution presumably reflects changes occurring in the subcellular sites of oxyradical formation—i.e. during the greening process photosynthetic reactions become more dominant in cell metabolism, necessitating an increase in chloroplastic SOD (as discussed below). During subsequent growth to maturity, SOD activities appear to change little. However, it has been observed that expression of the different enzymes is to some extent determined by the availability of their metal cofactors (reviewed in 48).

As the plant senesces, activity of all the SOD enzymes, together with that of other oxygen-detoxifying enzymes such as catalase and glutathione reductase, decreases (51, 151, 158, 207; C. Bowler, unpublished results). Processes that enhance the formation of oxyradicals and initiate lipid breakdown, such as lipoxygenase enzymes, are stimulated in senescing plant tissue (125), and the addition of hydrogen peroxide or hydrogen peroxide-generating compounds to excised rice leaves promotes senescence (149). These observations are consistent with the proposal that free radicals play an important role in senescence and ageing processes (see 3, 34, 132, 210). For example, in the fungus *Neurospora crassa*, conidial longevity is positively correlated with superoxide dismutase, catalase, and glutathione peroxidase activities (144); in *Drosophila*, a null mutation of Cu/ZnSOD results in a reduction in lifespan (157).

Regulation of SOD genes also appears to be very sensitive to environmental stress, presumably as a consequence of increased oxygen radical formation. This section documents the evidence for the generation of oxidative stress during different types of environmental adversity and summarizes the corresponding regulation of SOD enzyme activities which have been observed.

### *Photoinhibition*

The production of hydrogen peroxide by illuminated chloroplasts was first demonstrated by Mehler in 1951 (133). It has subsequently been shown that almost all of this hydrogen peroxide is derived from superoxide formed by the univalent transfer of electrons to oxygen from the electron acceptor of photo-

system I, and more particularly from ferredoxin (reviewed in 11, 78, 79). Since ferredoxin normally passes its electrons to NADP (via ferrodoxin-NADP reductase), the amount of superoxide formed in this side reaction is to some extent governed by the amount of NADP available, which in turn depends on the activity of the Calvin cycle and the supply of $CO_2$. Hence the amount of potential oxidant stress is dependent upon the photosynthetic state of the chloroplast. In general, superoxide radicals are more likely to be formed during periods of high photosynthetic activity; disturbance of normal photosynthetic reactions increases this likelihood even further.

Such correlations are reflected in the behavior of chloroplast SOD. mRNA levels of the FeSOD of *Nicotiana plumbaginifolia* were not significantly affected by diurnal fluctuations of light and dark, but when plants were kept in the dark for three days prior to illumination, FeSOD mRNA levels increased dramatically in response to light (213). This induction was not mediated by phytochrome and could be reduced by adding 3-(3,4-dichlorophenyl)-1,1'-dimethylurea (DCMU), a herbicide that blocks electron transport in photosystem II, thereby blocking superoxide production from photosystem I (10). These results indicate that chloroplastic SOD responds not directly to light but to the increased superoxide formation arising from the inefficient transfer of electrons through the photosystems owing to inadequate maintenance of the photosynthetic apparatus during the prolonged dark period.

In addition to superoxide and hydrogen peroxide (and therefore the potential to form hydroxyl radicals), illuminated chloroplasts can produce singlet oxygen by a transfer of excitation energy from chlorophyll to oxygen. Carotenoids can ameliorate this problem because they can react with singlet oxygen at diffusion-limited rates and can also quench the excited triplet states of chlorophyll that lead to singlet oxygen formation (reviewed in 11, 78, 79, 113).

During normal conditions chloroplasts are likely to be well adapted for minimizing the damage that can occur from misuse of photosynthetic energy transfer. Thylakoid membranes are rich in antioxidants such as $\alpha$-tocopherol and carotenoids, and the presence of SOD and ascorbate peroxidase provides an efficient enzymatic means for eliminating potentially harmful superoxide and hydrogen peroxide. In addition, the levels of these antioxidants can increase if light intensity increases slowly (69). However, because of the continuous absorption of light energy by the photosynthetic machinery, any perturbation of electron transport can lead to the donation of electrons to the wrong electron acceptor. If this happens to be oxygen, reactive oxygen species can be generated. Such disturbances can be caused by herbicides that interfere with electron transport or $CO_2$ fixation (see below) and also during conditions of photoinhibition, in which the absorbed light energy exceeds the capacity of the photosystems to direct it through photosynthetic electron



transport. Such a condition can arise during high light intensities and also when a temperature stress (chilling or heat) accompanies illumination.

Exposure of plants to high light intensities leads to a reduction of photosynthetic capacity owing to the redirection of photon energy into processes that inhibit photosynthetic capacity. Maintained long enough, this condition leads to the destruction of photosynthetic pigments (commonly referred to as photooxidation). While this pigment bleaching is dependent upon oxygen as well as light and appears to be mediated to some extent by reactive oxyradicals, the reduced photosynthesis that precedes it can occur largely in the absence of oxygen, hence questioning any involvement of active oxygen species (for reviews, see 11, 40, 70, 114, 159). Under such conditions photosystem II is the primary site of damage, most likely because of the destruction of the 32-kDa $Q_B$ polypeptide within the reaction center. Among other things, oxygen radicals have been implicated in this phenomenon (170, 195). Damage to photosystem I is usually less significant, but its appearance is dependent upon electron flow from photosystem II and upon the presence of oxygen (179). This observation may suggest that the formation of superoxide from oxygen by photosystem I or ferredoxin is the initial event in photosystem I damage. The iron-sulfur centers of photosystem I appear to be the sites of damage (98). Further evidence for these oxygen-dependent and -independent events have been obtained from experiments with isolated spinach chloroplasts or thylakoids, which showed that the addition of SOD or catalase could only provide partial protection against photoinhibitory conditions (16, 219). Nonetheless a biotype of *Conyza bonariensis* possessing elevated levels of chloroplastic SOD, glutathione reductase, and ascorbate peroxidase was reported to be resistant to photoinhibitory light (102).

Injury resulting from the combination of light with cold temperatures appears to bear some similarity to that described above, but in addition the peroxidation of membrane lipids is more pronounced (138, 220, 221). Indeed, the extent of this membrane damage may well govern chilling sensitivity because in the blue-green alga *Anacystis nidulans* genetic manipulation of fatty acid desaturation alone can result in alterations of chilling susceptibility (216). The effects of chilling are greatly exacerbated by light (e.g. 68, 152), and reactive oxygen species have been implicated in the destruction of lipids and photosynthetic pigments that occurs (186, 220, 221).

Consistent with the observations that oxygen radicals play some role in the cellular damage occurring as a result of photoinhibition, some reports document changes in SOD activity. In *A. nidulans* the onset of death by photoinhibition was more apparent when cellular SOD activity had been decreased by prior incubation in an atmosphere of nitrogen (1). In another blue-green alga, *Plectonema boryanum*, a mutant was isolated that was resistant to photooxidation (199). During exposure to photooxidative con-

ditions the SOD activity remained constant in the resistant mutant but dropped more than ten times in the sensitive parent strain. The maintenance of SOD activity was due to an increased synthesis of the thylakoid membrane–bound MnSOD and not to the soluble FeSOD. It was proposed that the increased synthesis of a hydrogen peroxide-insensitive SOD (the MnSOD) was important for maintaining high SOD activity during light-mediated stress because the $H_2O_2$ generated during photosynthesis may inactivate the sensitive FeSOD enzyme. This same adaptation has been reported for a chilling-resistant strain of *Chlorella ellipsoidea* (38).

In higher plants the resistance of cold-hardened spinach to subsequent chilling-mediated photoinhibition may be due to adaptation of the photosystems (194), but it also correlates with increased SOD activity and increases in the levels of enzymes of the Halliwell-Asada pathway that scavenge $H_2O_2$ in chloroplasts (182, 183). A new SOD enzyme was detected in protein extracts from cold-hardened plants that behaved the way a Cu/ZnSOD does in inhibitor tests with KCN and $H_2O_2$, but its cellular location was not determined. In addition, the ratio of carotenoids to chlorophyll increased, providing an additional means of scavenging reactive oxygen species. Analysis of chilling effects in chilling-sensitive tomato plants indicated that chloroplastic SOD was irreversibly inactivated, perhaps as a consequence of increased $H_2O_2$ generation. This inactivation was proposed as the reason for the enhanced lipid peroxidation (138). These results, together with those obtained from lower photosynthetic organisms, may therefore suggest that a combination of chilling with light leads to increased $H_2O_2$ formation that may eventually inactivate the chloroplastic SOD enzymes (Cu/ZnSOD and/or FeSOD).

Sunscald is a phenomenon related to photoinhibition caused by a combination of light and heat. It can severely affect the marketability of many kinds of fruits, flowers, and vegetables grown in warm climates. Oxygen radicals appear to be responsible for the damage done to photosystems and membranes (198). The tolerance of tomato, cucumber, and pepper fruit to sunscald at different stages of their development correlates with levels of carotenoids and with SOD but not peroxidase activity (165–167). Additionally, the artificial tolerance that can be induced experimentally in green tomatoes by controlled heat treatment parallels increases in SOD activity (165). Whether these changes in SOD activity were due to changes in chloroplastic, cytosolic, or mitochondrial SOD was not tested. However, in leaves of *N. plumbaginifolia* plants subjected to heat shock, only cytosolic Cu/ZnSOD mRNA levels increased appreciably, not those of MnSOD or FeSOD, and this induction occurred independently of light (213). A different situation is found in plants exposed to chilling conditions in combination with light. In this case only the chloroplastic FeSOD mRNA level increased during photoinhibitory conditions (213). However, if the plants were subsequently returned to ambient



temperatures increases of the MnSOD and cytosolic Cu/ZnSOD mRNA levels were observed. These different patterns of SOD induction mediated by chilling or heat shock in the light might thus imply that the mechanisms of photoinhibition are different in each case.

### *Paraquat and Other Herbicides*

Any perturbation in photosynthetic activity can cause the formation of reactive oxygen species initiating either from photosystem I, ferredoxin, or excited chlorophyll. Hence, herbicides that directly affect chloroplast activity can stimulate processes that induce damaging oxygen species. Herbicides that block photosynthetic electron transport, such as monuron, ioxynil, and atrazine, allow excitation energy to be transferred from chlorophyll to the carotenoids, which will be damaged progressively as a consequence. Once they are destroyed, the light energy may be transferred to oxygen, generating singlet oxygen and other species that can initiate lipid peroxidation (78, and references therein). Similarly, herbicides that act by inhibiting carotenoid synthesis, such as aminotriazole, metflurazone, fluridone, norflurazon, and pyrichlor, eliminate an important quencher of excitation energy, thus potentiating formation of singlet oxygen together with other reactive oxygen species (78, 113).

Redox-active herbicides such as the diphenyl ethers (e.g. acifluorfen) can also act through the production of reactive oxygen. These compounds cause the accumulation of photodynamic tetrapyrroles that produce reactive oxygen species (127). Levels of ascorbate and glutathione, together with enzymes of the Halliwell-Asada pathway, catalase, and peroxidase decreased in acifluorfen-treated cucumber cotyledons, decreases that were accompanied by lipid peroxidation (110). In contrast, however, Schmidt & Kunert (181) found that ascorbate, glutathione, and glutathione reductase activity increased. These differences may be due to the time at which the phenomena were investigated. In the former case the material may have been in the throes of death while in the latter the plant may have still been actively defending itself against the herbicide. In another report, protection against injury caused by acifluorfen could be obtained by pretreatment with $\alpha$-tocopherol (148).

Bipyridyl herbicides such as paraquat and diquat increase oxidative stress directly by generating oxygen radicals. Of the two, paraquat has been the most extensively studied; both appear to mediate identical effects. Also known as methyl viologen (1,1'-dimethyl-4,4'-bipyridinium chloride), paraquat is a redox-active compound that is photoreduced by photosystem I and subsequently reoxidized by transfer of its electrons to oxygen, forming the superoxide anion (reviewed in 11, 76, 162). Highly reactive hydroxyl radicals and related species produced from this superoxide are presumably the agents that cause cellular damage (12). Paraquat is also toxic to nonphotosynthetic

organisms because it can take electrons from many sources, in particular from NADPH-dependent diaphorases (84) and from a microsomal NADPH cytochrome P-450 reductase (79). This mechanism can probably occur in plants too, although the high rates of electron transfer through the photosystems during illumination ensure that photosystem I is the primary donor and that the effects of paraquat in the light are much more pronounced than in the dark.

Owing to its nonselective toxicity paraquat is now banned as a herbicide in most countries. However, the way it produces superoxide radicals has led to its experimental use for studies of oxygen toxicity in many organisms. In illuminated plants, paraquat causes a rapid inhibition of carbon dioxide uptake, followed by lipid peroxidation, the cessation of photosynthetic electron transport, and the breakdown of chlorophyll (25, 26, 37, 57, 81, 146, and references therein).

SOD has often been correlated with the mechanism of paraquat survival. In *E. coli*, which contains both MnSOD and FeSOD, only the former enzyme is induced by paraquat (83). Paraquat in fact induces about 40 proteins in *E. coli*, most of which have not been identified, including antioxidant and repair enzymes. Some of these are positively regulated at the transcriptional level by a gene product of the *soxR* locus (71, 212). The importance of SOD in paraquat survival has been shown by the isolation of a SOD-deficient mutant that is hypersensitive to paraquat (30).

When the green alga *Chlorella sorokiniana* is grown in sublethal concentrations of paraquat, SOD activity increases owing to the synthesis of a new MnSOD isozyme (161). This induced MnSOD activity, together perhaps with other protective enzymes, confers resistance to higher doses of the herbicide. Similarly, treatment of duckweed (*Spirodela oligorrhiza*) with benzyl viologen, a less-reactive bipyridinium compound, increased SOD activity slightly, which may have been a factor in the plant's subsequently observed resistance to paraquat (123). *Dunaliella salina* responds to paraquat by a general induction of SOD and catalase isozymes (164).

The effects of paraquat on the endogenous SOD enzymes in illuminated plants have been studied in several cases. Treatment of *Phaseolus vulgaris* (37) and *Lemna* (196) leaves caused a general increase in SOD activity. A study of the expression of the cytosolic and chloroplastic Cu/ZnSODs of tomato showed that mRNAs for both were induced by paraquat, although the former enzyme was the most strongly affected (R. Perl-Treves and E. Galun, personal communication). In *N. plumbaginifolia*, chloroplastic, cytosolic, and mitochondrial SOD expression was analyzed at the mRNA level, and all three were strongly induced by paraquat; but in this case the cytosolic Cu/ZnSOD was the least affected (213). These differences are probably a reflection of the different light intensities used in each experiment because



this can greatly influence the sites from which electrons are donated to paraquat. In this context, although Tsang et al (213) observed induction of all SODs in the light, paraquat treatment in darkness led only to an induction of cytosolic Cu/ZnSOD expression. In maize, however, the chloroplastic Cu/ZnSOD was induced in addition to the cytosolic Cu/ZnSOD by dark-incubation with paraquat (128), suggesting that there may also be differences between plant species.

Following the intensive use of paraquat in certain areas of the world several paraquat-resistant weeds evolved (121). Biochemical analysis of these has allowed further insights into the mechanisms by which plants protect themselves against oxidative stress. Harper & Harvey (80) analyzed SOD, catalase, and peroxidase activities in four paraquat-tolerant and eleven paraquat-susceptible cultivars of perennial ryegrass (*Lolium perenne*) and found that constitutive activities of SOD, catalase, and sometimes peroxidase were higher in all the paraquat-tolerant lines than in the susceptible lines. All of the increased SOD activity was associated with the chloroplasts. Similarly, a resistant biotype of *Conyza bonariensis* contained constitutively elevated levels of chloroplastic SOD, glutathione reductase, and ascorbate peroxidase (189). This variety was also reported to possess a mechanism of sequestration that prevented paraquat from entering the chloroplasts (64), but these results have been questioned (190). Genetic analysis of the variety indicates that the elevated activities of the three Halliwell-Asada pathway enzymes cosegregate, implying that one dominant nuclear gene is responsible for their control (187). In contrast, the mechanism of paraquat resistance in a variety of barley grass (*Hordeum glaucum*) is apparently not due to increased activities of these oxygen-detoxifying enzymes but may rather be related to uptake of the herbicide into the cell (160).

Paraquat-resistant plant varieties have also been artificially selected under experimental conditions. Paraquat-resistant calli of tobacco were obtained by three successive screenings on paraquat-containing media (65); these contained constitutively enhanced SOD but not catalase or ascorbate peroxidase activities. All of the increased activity was due to Cu/ZnSOD, as shown by inhibitor studies, but it could not be inhibited by antibodies against chloroplastic Cu/ZnSOD, suggesting that it was a cytosolic isoform. Thus in callus material, which is not actively photosynthesizing, the main site of the oxidant stress generated by paraquat may be in the cytosol. Plants regenerated from such calli remained resistant to paraquat, even though the chloroplast is likely the chief site of superoxide formation in such material. Resistant tobacco plants were also successfully regenerated from paraquat-selected callus by Miller & Hughes (139). These contained elevated levels of catalase and peroxidase but not of SOD activities (94). The selection of paraquat-tolerant mutants of the fern *Ceratopteris richardii* led to the isolation of two

allelic mutants with recessive nuclear mutations (86, 87). Biochemical studies could identify no differences in the levels of ascorbate, glutathione, SOD, catalase, peroxidase, glutathione reductase, dehydroascorbate reductase, and ascorbate peroxidase activities in the presence or absence of paraquat (31); the uptake of paraquat was identical to that in the wild-type strain. A mutant with a mutation that enhances paraquat tolerance and is not linked to the other locus has subsequently been isolated (87, 88), but its effects on oxygen-detoxifying enzymes have not yet been studied.

In summary, paraquat has a strong influence on the expression of SOD and other oxygen-detoxifying enzymes, as would be predicted from its mode of action. However, the importance of other mechanisms is not precluded—for example, the resistance of one *E. coli* mutant was due to a decreased uptake of paraquat (108), and *E. coli* cells deficient in spermidine biosynthesis have an increased sensitivity to paraquat (140). Studies with paraquat-resistant mutants, together with the realization that more than 40 proteins are induced by paraquat in *E. coli*, demonstrate that much remains to be learned about the basis of an organism's defense against oxidative stress, even when using paraquat, the simplest model system available.

## *Atmospheric Pollutants*

Industrialization has increased considerably the concentrations of noxious chemicals such as ozone and sulfur dioxide in the lower atmosphere in recent decades. The effects of these air pollutants on the physiological processes of plants have been widely studied and recently reviewed (42). Ozone causes more damage to crops and forests than all other air pollutants combined. Although plant sensitivity varies greatly between species and with environmental conditions, the effects of these pollutants are similar: Photosynthetic activity is reduced whereas respiration is often increased. In the first place plant sensitivity can be governed by the stomatal conductance—i.e. whether the stomata are open or closed determines how much of the pollutant enters the leaf. Stress ethylene formation may also determine plant susceptibility to ozone (136). For this reason exposure to other stresses that induce ethylene—e.g. chilling, high light intensities, and other pollutants—may alter a plant's response to subsequent ozone exposure (see 134).

Lipid peroxidation similar to that occurring during senescence has been found in plants following ozone fumigation (150), but the importance of oxygen radicals is not known. When ozone was mixed with plant protoplasts no plasma membrane damage was observed (74), indicating that the mechanism of damage to plant cells is not simply mediated by the ozone molecule itself. In vitro treatment of DNA with ozone did not result in DNA damage (as measured by the formation of 8-hydroxyguanine, which is formed from the reaction of hydroxyl radicals with guanine bases) (59). Nonetheless exposure



of whole plants or isolated chloroplasts to ozone resulted in increased 8-hydroxyguanine formation, suggesting that hydroxyl radicals were being generated from the ozone. Indeed, ozone has been reported to degrade into superoxide, hydrogen peroxide, and hydroxyl radicals (90) and also to produce singlet oxygen from reactions with biological molecules (107). Conversely, lipid peroxidation and the breakdown of chlorophylls and carotenoids that occurred in leaf discs derived from ozone-fumigated spinach plants could be inhibited by the addition of superoxide radical scavengers (173).

An important new finding is that phytotoxic organic hydroperoxides (ROOH) are present in ozone-treated leaves of isoprene-emitting plants (such as spruce, pine, and fir) formed from the reaction of ozone with biogenic alkenes such as monoterpenes (85). In an independent study the concentration of monoterpenes present in Norway spruce decreased following ozone fumigation (115). Reactive oxygen species may be formed by intermediate reactions in ROOH formation; together with ROOH species, these may initiate the lipid peroxidation and other damage observed in ozone-stressed plants. Hydrogen peroxide is produced when ozone reacts with these terpenes (20).

Evidence for the direct involvement of oxygen radicals in $SO_2$ toxicity is more substantial. Its damage to biological systems is probably a result of radicals generated during its oxidation to sulfate, which include $O_2^{\cdot}$, $OH\cdot$, and $SO_3^{\cdot}$ (11). In plant chloroplasts, $SO_2$ oxidation can be initiated by superoxide generated from photosystem I (9). $SO_2$ fumigation of spinach leaves and chloroplasts caused the accumulation of $H_2O_2$ (203) that could be reduced somewhat by cytochrome C (which oxidizes $O_2^{\cdot}$ to $O_2$) and by SOD. This $H_2O_2$ formation apparently caused the inactivation of endogenous SOD, catalase, ascorbate peroxidase, and glutathione reductase activities, together with a reversible inhibition of Calvin cycle enzymes (204). Also, leaf homogenates derived from spinach leaves suffered lipid peroxidation and chlorophyll and carotenoid bleaching when exposed to $SO_2$. This damage could be inhibited by SOD and by free-radical scavengers (154, 155, 191).

Lipid peroxidation is likely a secondary consequence rather than a mediator of the toxicity of these pollutants. This idea is inferred from the fact that the inhibition of photosystem II activity (resulting in a reduction of photosynthesis) precedes lipid peroxidation, at least in $SO_2$-treated leaves of *P. vulgaris* (39). Other effects of these pollutants are the increased synthesis of ethylene, polyamines, phytoalexins, antioxidants, and defense-related proteins such as $\beta$(1,3)-glucanases (115, 135, 178), events that also occur during pathogen attack (discussed below) and other stress conditions, including senescence.

The participation of oxygen-detoxifying enzymes has been examined in several cases. Extracellular peroxidases were induced by ozone in both *Sedum*

*album* (33) and Norway spruce, but in the latter an extracellular SOD was also induced (32). In red spruce, loblolly pine, and Scots pine all SOD enzymes were induced (other enzymes were not assayed) (208). Ozone treatment of spinach leaves induced SOD, peroxidase, and catalase (47), whereas in *P. vulgaris* ozone resistance was correlated with enhanced SOD and catalase (not peroxidase) activities (122). However, ascorbate peroxidase was proposed to be a key enzyme governing ozone resistance in mung bean and pea (134).

MnSOD was induced by $SO_2$ in the green alga *Chlorella sorokiniana* and this resulted in subsequent resistance to paraquat (163). In poplar leaves however, Cu/ZnSOD and not MnSOD was induced by $SO_2$, with no change in peroxidase activities (207). In Scots pine, Norway spruce, and *Lemna* a general increase in SOD was noted (97, 196). Additionally in *Cicer arietinum* and *Vicia faba*, $SO_2$ tolerance correlated with total SOD activity of the leaves (4).

In contrast to these reports other workers have shown that neither SOD nor catalase responded to ozone or $SO_2$ in maize (129), and SOD was largely unaffected by ozone in *P. vulgaris* (36). Spinach leaves fumigated with ozone in fact exhibited decreased SOD and catalase activities, though ascorbate peroxidase activity increased (173, 205, 206).

Plant varieties with differing sensitivities to atmospheric pollutants provide a valuable tool for identifying the mechanism of phytotoxicity. In some cases, such as the ozone-sensitive tobacco variety BelW3, increased susceptibility may be due in the first place to stomatal behavior (141), although differences in polyamine biosynthesis, ethylene formation, and $\beta(1,3)$-glucanase synthesis have also been noted (116). The differential ozone sensitivities of the cultivars of *P. vulgaris* tested by Hucl et al (93) do not result from stomatal behavior, and the susceptibility of these cultivars was not correlated with the amounts of lipid-soluble antioxidants or SOD (131). Several cultivars of spinach with differing ozone sensitivities were examined, but no clear correlation was found between resistance and levels of ascorbate, glutathione, SOD, catalase, ascorbate peroxidase, or glutathione reductase (206). Of four ozone-tolerant cultivars of *Nicotiana tabacum* analyzed by Shaaltiel et al (188), only one contained increased activities of SOD and glutathione reductase—the only one that was also resistant to paraquat. In a reversed approach, Tanaka et al (202) examined the $SO_2$ and ozone sensitivity of tobacco plants (originally selected with paraquat) that contained up to five times the SOD of wild type, but normal ascorbate peroxidase and glutathione reductase activities. These plants were resistant to $SO_2$ but not to ozone. Varieties of *Conyza bonariensis*, *Lolium perenne*, and maize with increased activities of the enzymes of the Halliwell-Asada pathway were more tolerant to $SO_2$ than parental varieties (126, 188).

To summarize, evidence for an important function of SOD in plant protection to $SO_2$ is fairly convincing, although the evidence for its involvement in ozone resistance is often indirect, fragmentary, and inconclusive. Much of the inconsistency can be ascribed to differences in experimental conditions used by different researchers. Small differences in growth conditions might have profound effects on the protection mechanisms against oxidative stress. Furthermore, the levels of certain enzymes, such as SOD, appear to be under developmental control (C. Bowler & D. Hérouart, unpublished results). Finally, the use of inefficient methods to filter the air can mean the mixing of pollutants already present with those administered, which can have drastic effects on the outcome of the fumigation, as was convincingly shown in experiments where the effects of ozone alone, $SO_2$ alone, and a combination of both were compared (147). These problems have now been acknowledged and it is to be hoped that the phytotoxic mechanisms of air pollutants will become better understood.



### *Waterlogging and Drought*

Waterlogging imposes an oxygen shortage on submerged plant organs. The plant responds by altering its pattern of protein synthesis. The expression of pre-existing proteins is repressed, and a new set of proteins is synthesized, the so-called anaerobic polypeptides (ANPs). Alcohol dehydrogenase has been the most extensively studied ANP (reviewed in 172). Many plants are able to survive this period of anoxia only to die upon subsequent re-exposure to air, suggesting that oxidative damage may occur during the recovery phase. Indeed, lipid peroxidation was found to be much higher in rhizomes of the flooding-sensitive *Iris germanica* than in rhizomes of the tolerant *Iris pseudacorus* after their post-anoxic exposure to air (95). A dramatic increase in total SOD activity (up to 13-fold) occurs during the anoxic phase in the resistant but not in the sensitive variety (142). Most of this increase is due to Cu/ZnSOD, but it was not determined where this enzyme was localized within the cell. It was suggested that this increased SOD activity was vital in protecting the plants against the oxidant stress generated upon re-exposure to air. This phenomenon may be analogous to the post-ischemic reperfusion injury that occurs in animal tissues (see 79). When oxygen is re-introduced to an ischemic or hypoxic tissue, further damage occurs that is mediated in part by oxygen radicals. The addition of SOD prior to oxygenation can sometimes protect against the subsequent damage.

A plant's response to drought stress is a complex phenomenon that appears to involve the synthesis of polyamines and a new set of proteins whose function is largely unknown (reviewed in 29). Abscisic acid is central in the response because it stimulates stomatal guard cells to close, reducing water loss. This process also reduces the availability of $CO_2$ for photosynthesis, which can lead to the formation of reactive oxygen species from the misdirect-

ing of electrons in the photosystems. Hence, mechanisms that reduce oxidative stress may play a secondary role in drought tolerance.

In tomato, cytosolic Cu/ZnSOD was induced strongly by drought while the chloroplastic Cu/ZnSOD remained largely unaffected (R. Perl-Treves and E. Galun, personal communication). Glutathione reductase activity increased in drought-stressed wheat and cotton plants (27, 66), and it was proposed that, as well as removing $H_2O_2$, this increase may make NADP available that can accept electrons from ferredoxin, thereby minimizing superoxide formation. In drought-tolerant *Hordeum* species, levels of glutathione reductase and ascorbate peroxidase increased, but SOD activity was not examined (192). Drought-stressed cotton was found to be resistant to a subsequent challenge of paraquat (27), which may indicate the existence of a common protective mechanism against these stresses.

Drought-induced changes in lipid peroxidation and the activities of SOD and catalase were compared in two mosses, the drought-tolerant *Tortula ruralis* and the drought-sensitive *Cratoneuron filicinum* (50). During stress treatment the drought-tolerant moss showed lower levels of lipid peroxidation, together with increased levels of both enzymes; the opposite occurred in the sensitive moss. Glutathione metabolism was subsequently studied in the tolerant moss, and oxidized glutathione (GSSG) was found to be a good indicator of drought stress (49). Drought-tolerant and -intolerant maize inbreds were analyzed by Malan et al (126), and resistance was found to correlate with Cu/ZnSOD and glutathione reductase activities, although higher levels of one enzyme alone apparently did not confer drought tolerance. These varieties were also tolerant to paraquat, acifluorfen, and $SO_2$, again suggesting the involvement of common defense mechanisms.

## *The Defense Response to Pathogens*

The two different interactions observed between plant and pathogen have been classified as incompatible and compatible. Incompatible reactions (resistant host, avirulent pathogen) are often characterized by the appearance of a hypersensitive response, a localized necrosis of plant cells at the penetration site of the pathogen, which prevents further spread to other cells. The death of the plants' own cells is not observed in a compatible interaction (susceptible host, virulent pathogen), in which the pathogen can spread to other areas of the plant. The hypersensitive response involves a multitude of biochemical events in the plant, including the synthesis of ethylene and phytoalexins; the reinforcement of cell walls with callose, lignin, and related compounds; the accumulation of cell wall-bound hydroxyproline-rich glycoproteins; and the synthesis of the pathogenesis-related (PR) proteins that include glucanases, chitinases, peroxidases, and proteinase inhibitors (reviewed in 52). The peroxidases play an important extracellular role in strengthening the cell wall,



and hence their involvement in intracellular defense against oxidative stress may be minimal. Disease resistance genes of unknown function also exist, which are hypothesized to encode important proteins in signal transduction pathways.

Reactive oxygen species may also play a role in these events. Increased lipid peroxidation and, in some cases, increased lipoxygenase activity have been reported in bacteria-induced hypersensitive reactions of cucumber, tobacco, and *Phaseolus vulgaris* (2, 41, 112), in cowpea infected with cucumber mosaic virus (109), in tomato leaves treated with elicitor from *Cladosporium fulvum* (153), and in a bacteria-induced hypersensitive response in tobacco cell suspensions (111). Lipid peroxidation also accompanied the biosynthesis of phytoalexins in cultured bean cells treated with an elicitor from the fungal pathogen *Colletotrichum lindemuthianum* (171). Furthermore, Rogers et al (171) showed that lipid peroxidation and phytoalexin biosynthesis could be stimulated in the bean cells by adding generators of oxygen radicals (xanthine and xanthine oxidase, which produce superoxide; and chelates of iron, which promote hydroxyl radical formation). It was nevertheless not possible to block elicitor-induced events by the addition of the free-radical scavengers mannitol, tiron, or benzoate. Perhaps the ability of the elicitors to produce oxygen radicals exceeds the scavenging capacities of the compounds added; dose/response curves in which the concentrations of elicitor and scavenger were varied were unfortunately not presented. In contrast, Epperlein et al (58) observed that the elicitation of phytoalexins in legumes could be prevented by adding oxyradical scavengers. Also, the lipid peroxidation observed in cucumber cotyledons and in tobacco plants and cell suspensions induced by incompatible bacteria could be delayed but not prevented by adding free-radical scavengers (2, 111, 112). The inability of singlet oxygen scavengers to affect the hypersensitive response in tobacco cell cultures may suggest that singlet oxygen is not involved in the response (175).

The use of membrane-associated fluorescent dyes to monitor elicitor effects on cultured plant cells demonstrated that major changes in membrane permeability occur within minutes of elicitor addition (124). It was subsequently shown that an oxidative event, inhibitable by catalase but not by SOD, was responsible for these modifications, implying the involvement of $H_2O_2$ but not superoxide (5). It was proposed that the elicitor may somehow stimulate a plasma membrane–bound oxidase to transfer two electrons and two protons to oxygen, forming hydrogen peroxide that may then act as a second messenger to initiate the defense response of the cell. The role of $H_2O_2$ as a second messenger for hormones such as insulin in animal cells has received much attention (reviewed in 168). Evidence is also accumulating for the existence of a superoxide-generating NADPH-oxidase bound to the membranes of plant cells that is stimulated immediately after invasion of cells by an incompatible

pathogen. Such a phenomenon has been reported in incompatible reactions of tobacco (2, 54, 111), of potato with *Phytophthora infestans* (35, 53), in reactions of the rice blast fungus with rice (106, 185), and in tomato and pea cultivars infected with nematodes (223, 224, 226). The generation of superoxide in the reaction of potato with elicitors from *P. infestans* has, however, been questioned (143).

Since these events occur earlier than any others so far discovered, these observations suggest that superoxide, hydrogen peroxide, or both are intimately involved in determining the outcome of plant/pathogen interactions. As for their function, three likely possibilities exist: 1. Because $H_2O_2$ is a substrate for many important reactions in cell wall reinforcement, its availability may govern the ability of the pathogen to penetrate into the plant cell; 2. superoxide or hydrogen peroxide may be second messengers (see the section on SOD regulation, below); and 3. superoxide and/or hydrogen peroxide may be actively involved in killing pathogen and/or host cells in the hypersensitive reaction. This third possibility invokes a mechanism remarkably similar to that used by mammalian phagocytes, which generate superoxide by means of plasma membrane-bound oxidases; the superoxide is then converted to $H_2O_2$ and other reactive oxygen species, facilitating bacterial killing (14, 96).

If any of these explanations is correct, the enzymes involved in oxyradical scavenging play a critical role in determining the consequences of plant/pathogen interactions, and their subcellular (or extracellular) location is likely very important. Certain findings suggest that this is the case. For example, in tomato roots infested with a compatible race of the nematode *Meloidogyne incognita*, SOD activity increased considerably compared to that in roots of resistant varieties that displayed the hypersensitive response and did not induce SOD activity (222, 224). The same relationships have been noted in compatible and incompatible reactions of pea and potato to nematodes (6, 225).

In support of the possibility that reactive oxygen species are involved in cell killing, these observations might be interpreted to indicate that in an incompatible reaction the low SOD activity allows superoxide and related oxygen species to destroy plant cells (via processes such as lipid peroxidation), leading to the hypersensitive response, while in a compatible reaction the high plant SOD activity ensures detoxification of the oxyradicals and hence no localized cell death. However, it is not clear how an increase in SOD activity, which is likely to be almost entirely intracellular, could prevent damage by oxygen species generated extracellularly. If either superoxide or hydrogen peroxide were a second messenger, on the other hand, the changes in SOD activity could modify their signal transducing capabilities by controlling quantities within the cell in compatible and incompatible reactions. In such a case, a cytosolic SOD would likely be the most important enzyme. Unfortunately, it is not yet clear which of the SODs is responsible for the increase in activity occurring during a compatible reaction. Mitochondrial MnSOD was induced by an incompatible reaction between *N. plumbaginifolia* and *Pseudomonas syringae* (23), but this induction most likely reflected the increased oxidant stress generated by increased mitochondrial activity that occurs during infection.



SOD may play a more direct role in the defense of plants against fungi of the genus *Cercospora*, a large group of fungal pathogens that cause damaging leaf spot diseases on a wide range of economically important crops. These fungi produce cercosporin (1,12-bis(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone), a nonspecific phytotoxin sensitized by light to produce both singlet oxygen and superoxide (46). Altertoxins (4,9-dihydroxyperylene-3,10-quinones), similar in structure to cercosporin, are produced by plant-pathogenic fungi of the *Alternaria* genus, and are photosensitized to produce superoxide in a fashion like that of cercosporin (82). Cercosporin causes light-dependent peroxidation of plant membrane lipids, presumably mediated by toxic oxygen species (43, 45). In maize, catalase activity increased upon exposure to cercosporin in the light, although SOD changed little, with the possible exception of the mitochondrial MnSOD (180). Although plant varieties have been selected that are resistant to the toxin, there are as yet no biochemical data on the nature of the resistance mechanism. However, a paraquat-resistant tobacco variety containing elevated levels of SOD but not catalase or peroxidase (65) is resistant to cercosporin, while another with increased activities of catalase and peroxidase but not SOD (94) is as sensitive to cercosporin as the wild type (44).

## *The Phenomenon of Cross-Tolerance*

Tolerance to certain environmental stresses can clearly arise by several possible mechanisms, each likely to involve pleiotropic effects, and a biotype tolerant to one condition can also be tolerant to others. SOD is one component that can determine this cross-tolerance. Such determination was first observed in the unicellular green alga *Chlorella*. Prior growth of *C. ellipsoidea* in sublethal concentrations of paraquat (which induces MnSOD activity) can decrease the injury resulting from chilling-mediated photoinhibition (38); conversely, growth of *C. sorokiniana* in the presence of sulfite increases MnSOD content and confers resistance to paraquat (163). Such phenomena have since been reported for several plants (89, 101, and references therein; 202); Table 1 summarizes the cross-tolerances now known. Although the level of cross-tolerance observed may not be sufficient in all cases to be of agronomical value, it may be of practical use since it is easier to test leaf discs

for paraquat tolerance than it is to assess a complex trait such as drought tolerance. These results also reveal that many diverse stresses produce similar effects at the cellular level, one component of which is oxidative stress. The mechanism of this phenomenon may involve ethylene, known to be formed during many stress conditions. Ethylene pretreatment of mung beans, for example, conferred protection against a subsequent exposure to ozone, hydrogen peroxide, and paraquat (134). It is interesting that ethylene induces plant MnSOD (23).

### *The Mechanism of SOD Regulation*

SOD activity is induced by diverse stress conditions. At first glance it is logical to assume that certain common components of these stresses are the chief mediators of SOD gene regulation. In *N. plumbaginifolia*, mitochondrial MnSOD responds to increased oxyradical formation in the mitochondria while chloroplastic FeSOD responds to such an event occurring in the chloroplasts (23, 213). Cytosolic Cu/ZnSOD probably responds to cytosol-localized reactions in a similar fashion. The effect of a particular stress on SOD gene expression is thus likely to be governed by the subcellular sites at which oxidative stress is generated.

Because the genes encoding the SOD enzymes are clearly not coregulated, how may such responses be mediated? The ubiquity of superoxide and hydrogen peroxide suggests that they do not themselves direct the diverse profiles of SOD gene expression. The OxyR protein of *S. typhimurium*, a transcriptional regulator of hydrogen peroxide-inducible genes, activates these genes only when it has been oxidized (201). The *sox*R gene product of *E. coli* (71, 212) is probably regulated in the same way. Reactive oxygen

**Table 1** Summary of the cross-tolerances found in different plant cultivars

| Species | Selection | Cross-tolerance | Enzymes involved | References |
|---|---|---|---|---|
| *Ceratopteris* | acifluorfen | paraquat | ? | 89 |
| *Conyza bonariensis* | paraquat | atrazine acifluorfen, $SO_2$, photoinhibition | SOD*, GR, AP | 102, 188 |
| *Gossypium hirsutum* | drought | paraquat | GR | 27 |
| *Lolium perenne* | paraquat | $SO_2$ | SOD, GR | 188 |
| | $SO_2$ | paraquat | SOD, GR | 188 |
| *Nicotiana tabacum* | ozone | paraquat | SOD, GR | 188 |
| | paraquat | $SO_2$, *Cercospora* | SOD | 44, 202 |
| *Zea mays* | drought | paraquat | SOD, GR | 126 |
| | | $SO_2$, acifluorfen | | |

*Abbreviations: AP, ascorbate peroxidase; GR, glutathione reductase; SOD, superoxide dismutase.



compounds have also been implicated in the regulation of transcription in eukaryotic cells; a recent report presents evidence that the regulation of the transcription factor NF-$\kappa$B by a wide range of diverse agents (such as TNF-$\alpha$, calcium ionophores, interleukin-1, and phorbol esters) is mediated through reactive oxygen species (184).

How the observed complexity of SOD regulation in plants could be controlled simply by the oxidation/reduction state of a single transcription factor is not clear. However, specific regulation could be achieved if the signaling factor regulating each class of SOD were generated in specific compartments. To induce expression of the required SOD, this molecule would then need to transfer quickly from the chloroplast or the mitochondrion to the nucleus because all SODs are encoded there. The required specificity could be achieved if small molecular components specific to chloroplasts, mitochondria or cytosol could be the primary sensors and signal transducers of compartment-specific stress.

Lipid-derived molecules could serve in this role. Fatty acids specific for chloroplastic, mitochondrial, or plasma membranes could be cleaved by an oxidative event, leading to the release of a hydrophilic molecule that could diffuse to the nucleus and interact with particular transcription factors to activate the gene encoding the required SOD enzyme. Such a fatty acid derivative could meet the requirements for a signaling molecule: It would be small, specific, readily modified by reactive oxygen, and diffusible. Several examples of biologically active lipids from mammalian systems exist, including the prostaglandins, leukotrienes, and lipoxins, which originate from the oxidation of fatty acid derivatives initially cleaved from membrane lipids by phospholipases or lipoxygenases (79, 176).

Genes that regulate SOD expression have not yet been isolated from any eukaryotic species. In plants these may eventually be isolated by methods requiring promoter analysis, gel shift assays, DNase I footprinting, and the screening of expression libraries with DNA sequence motifs known to be recognized by the factor of interest. Alternatively plant mutants may be used to isolate regulatory genes by genetic approaches. Although several plant mutants have been described that have mutations in regulatory genes controlling SOD expression—e.g. *Conyza bonariensis* (187) and *Lolium perenne* (80)—the lack of good genetic maps in these species make them currently worthless for isolating the regulatory genes themselves. Soybean varieties have been described that have variant patterns of SOD activities when visualized on polyacrylamide gels, although the mutations are likely to be in structural genes encoding SOD and not in regulatory genes (73, and references therein). In maize, which has excellent genetic systems available, similar mutations were also found in some varieties (19); but in addition one strain was found that expressed reduced levels of the three Cu/ZnSODs but

normal levels of MnSOD. It was proposed that a regulatory mutation may be responsible (18). The character was inherited as a recessive trait and was probably polygenic in nature.

Some of these plant varieties may be of use in biochemical approaches to study SOD regulation. For example, by studying the proteins that bind to the promoters of different SOD genes using nuclear extracts derived from mutant and wild-type varieties it may be possible to identify particular protein factors that are present in much greater amounts in one variety or that have altered expression patterns—e.g. constitutive in the mutant and inducible in the wild type. Such approaches may lead to the identification of the most interesting factors since their mutation was clearly shown a priori to affect SOD expression.

## GENETIC ENGINEERING OF SOD IN PLANTS

The induction of SOD in response to the diverse environmental conditions discussed in the previous section indicates that it plays an important role in a plant's defense mechanism. It may be a central component, in which case its genetic manipulation might result in stress-tolerant phenotypes. Alternatively, the data collected may simply demonstrate the ubiquity of oxidative stress in plant processes. In this case alterations in SOD activity resulting from the overproduction (or underproduction) of SOD would perturb the normally optimized mechanisms. Although likely to lead to a better understanding of oxygen toxicity, such perturbation would probably be of little use agronomically.

It has been suggested that some of the stress-tolerant plant varieties analyzed with respect to SOD have acquired tolerance by increasing SOD activity alone. This is a gross simplification of the biochemical status of such varieties, and the number of differences found with respect to the parental line is often a simple reflection of how many parameters were studied. Hence, although biochemical analysis of such mutants may suggest a role of SOD, a true evaluation of the effects of changing SOD activity alone in plants can be obtained only by genetic engineering. To obtain a complete picture, different SOD genes should be overexpressed, because their enzyme products each have slightly different properties. In particular, the inactivation of Cu/ZnSOD and FeSOD by $H_2O_2$, their reaction product, and the contrasting resistance of MnSOD to $H_2O_2$ may be pertinent to their particular effects (as indicated below). Enzyme activity should also be augmented in one or more of the subcellular compartments because different stress conditions appear to affect them in different ways. Approaches that may result in reduced SOD activity, such as antisense (or ribozyme) technology, are now feasible and should be complementary to any overexpression studies.



### *Cu/ZnSOD and MnSOD Overexpression*

The first report of the genetic manipulation of SOD in plants described the generation of tobacco and tomato plants that overproduced a chloroplastic Cu/ZnSOD derived from petunia (209). Tobacco plants expressing maximal levels were studied for their susceptibility to light-mediated paraquat damage in leaf disc assays by measuring $^{14}CO_2$ assimilation, photosystem II fluorescence, and chlorophyll bleaching. Under these conditions, superoxide is likely to be formed almost exclusively within the chloroplasts owing to the high rate of electron transfer through the photosystems. The transgenic plants behaved no differently from plants that did not contain increased chloroplastic Cu/ZnSOD. Similarly, experiments to evaluate the effect of photoinhibitory conditions (chilling and high light) on transgenic tomato plants indicated no significant difference between the plants that produced elevated Cu/ZnSOD and control plants. It was concluded that the increased activity of SOD alone in the chloroplasts was not sufficient to provide protection against oxygen toxicity due to the increased $H_2O_2$ that it would create. The authors proposed that the genetic engineering of the chloroplastic $H_2O_2$-detoxifying system (the Halliwell-Asada pathway), in addition to SOD, may be necessary to produce a resistant phenotype. The effect of increased chloroplastic SOD activity on the activity of the endogenous pathway was not tested.

As might be predicted from studies with other organisms and from the biochemical properties of the SOD enzymes, overproduction of MnSOD produces different results. In our laboratory we overproduced a *N. plumbaginifolia*–derived MnSOD and targeted the enzyme either to the mitochondria or to the chloroplasts of tobacco cells (25). Leaf disc assays with paraquat were again chosen as the model system, and light-mediated damage was assessed by measuring membrane damage, photosystem II fluorescence, and the formation of pheophytin, a derivative of chlorophyll formed by the action of paraquat. Leaf discs derived from plants that contained increased mitochondrial MnSOD behaved much the way control material did, as would be predicted, since the major site of superoxide production is likely to be the chloroplasts in these experiments. However, overproduction of MnSOD in the chloroplasts conferred protection against paraquat toxicity that was correlated with the increases in its activity. We estimate that maximal activity levels of MnSOD in transgenic plants were comparable to those of the petunia Cu/ZnSOD in tobacco (209), which did not confer protection to paraquat. Hence differences in activity levels of the MnSOD and Cu/ZnSOD cannot explain the disparity in these results; rather, we believe the differing $H_2O_2$ sensitivities of the two enzymes is responsible.

We also studied the effect of paraquat on leaf discs maintained in complete darkness. Since photosynthetic electron transport does not operate in the dark, the relative proportion of mitochondrially generated superoxide should increase. However electron sources for paraquat do still exist in dark-

maintained chloroplasts, as was shown by Law et al (119). These may be components of a chlororespiratory electron transport pathway known to be stimulated in the dark in photosynthetic prokaryotes and green algae, because recent evidence suggests it is also present in plant chloroplasts (67, and references therein). The results of these dark experiments were markedly different from those of the light experiments. Small increases of either chloroplastic or mitochondrial MnSOD were detrimental for the plant cells while larger increases conferred resistance.

### *Prospects for Stress Tolerance through Genetic Engineering of SOD*

The results discussed above have some bearing on the contrasting effects of SOD seen in bacterial and animal systems, and indicate a fine line between benefit and injury arising from changes in SOD activity. We believe these results can be explained on the basis of the Haber-Weiss reaction (see above), the metal ion–catalyzed formation of highly reactive hydroxyl radicals (OH·) from the comparatively unreactive superoxide anion (the SOD substrate), and hydrogen peroxide (the SOD reaction product). Augmentation of SOD activity will change the $O_2^{\cdot}/H_2O_2$ balance within the cell, and this will either increase or decrease the likelihood of OH· formation (for discussion see 25). This will then determine whether the genetically engineered change in SOD activity has been beneficial or detrimental to the plant. Unfortunately, this balance between help and hindrance may be so easily crossed that no approach to stress tolerance engineering via SOD alone can succeed.

Nonetheless, if a plant's defenses against oxidative stress could be reinforced with new genes and coordinated to maintain the appropriate physiological balance of all the components, stress tolerance would likely improve, because free-radical formation is a ubiquitous component of environmental adversity. This may necessitate, as Shaaltiel & Gressel (189) first pointed out, the manipulation of the whole oxidant stress defense system. For plant chloroplasts this would mean increasing the levels of SOD, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase—quite a challenge for current plant molecular biology. Increasing the activity of all of these enzymes in transgenic plants would perhaps be best achieved by manipulating the regulatory processes that control their expression. In *Conyza bonariensis*, at least, one nuclear gene appears responsible for increasing the activity of all these enzymes (187), and thus its modification might produce such an effect. However, much work remains to be done before such genes can be isolated. Even this approach is not without its problems because the resulting perturbations in oxidized and reduced ascorbate, glutathione, and NADP are likely to generate additional consequences.

The only other approach that is currently accessible to plant molecular biologists is to overproduce a hydrogen peroxide–detoxifying enzyme alongside SOD. Catalase and peroxidase are the two possibilities, and catalase is likely the best choice because it does not require any substrates for catalysis. This self-sufficiency should ensure against perturbation in other cellular components. Such an approach would be best carried out by re-targeting catalase to chloroplasts, cytoplasm, or mitochondria in order to allow $H_2O_2$ removal at its site of formation. Also, the targeting of these enzymes to sites within the cell most at risk from oxidative damage—e.g. membranes—may be a further approach to decreasing oxygen toxicity (see 99). As our knowledge of oxidative stress will improve considerably by attempting such approaches it is possible that an optimized method for improving stress tolerance via the manipulation of a plant's defense system will be found, particularly as new methods become available for plant gene manipulation.



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# WIDE CROSSES IN CEREALS

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## CONTENTS



## INTRODUCTION

Cereals constitute a central part of the human diet. A steadily growing population and limited areas available for growing crops make it necessary to



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